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STUDIES ON STRUCTURAL AND FUNCTIONAL CHANGES OF MOUSE CHOROID PLEXUS IN THE INITIATION OF NEUROINFLAMMATION

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УНИВЕРЗИТЕТ У БЕОГРАДУ

БИОЛОШКИ ФАКУЛТЕТ

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ЗНАЧАЈ СТРУКТУРНИХ И ФУНКЦИОНАЛНИХ ПРОМЕНА ХОРОИДНОГ ПЛЕКСУСА МИША У ИНИЦИЈАЦИЈИ НЕУРОИНФЛАМАЦИЈЕ

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ABSTRACT

Neuroinflammation has been considered a common denominator and crucial player in neurodegeneration observed in various central nervous system (CNS) disorders. Considering the increasing evidence on the role of choroid plexus (CP) in neuroinflammatory processes through alterations in morphology and functionality of the choroid plexus epithelial (CPE) cells, the main goal of this thesis was to test the contribution of the CP in the initiation of neuroinflammation in two animal models of neuroinflammation-associated diseases: a model of Alzheimer's disease, induced by intracerebroventricularly (i.c.v.) injected amyloid beta oligomers (AβO) and a lipopolysaccharide (LPS) induced sepsis animal model of systemic inflammation. Main findings from this research conclude that blood cerebrospinal fluid barrier (BCSFB) permeability is increased upon AβO injection, resulting from the loss of typical cuboidal morphology of CPE cells and a decrease in expression of tight junctions components. In the CP, upregulation of gene expression for various cytokines was observed, as well as their increased levels in the cerebrospinal fluid (CSF). Also, increase in gene expression for several matrix metalloproteinases (MMP) in the CP, and in MMP activity in the CSF was noted. After i.c.v. injection of broad spectrum MMP inhibitor with AβO, prevention of AβO-induced BCSFB permeability was found. In accordance with this, increase in BCSFB permeability upon AβO injection was observed in MMP3 deficient mice, but to a lesser extent. Furthermore, an increase in the number of particles in the CSF and an increase in gene expression of extracellular vesicles (EV) markers and miR-155 was found in the CP. A similar pattern of changes in the CP was observed in response to LPS injection compared to AβO injection. The results of this study revealed a significant role for the CP in the initiation of neuroinflammation, through structural and functional changes, in two different models associated with neuroinflammation.

KEY WORDS: BCSFB, CP, MMP, EV, AβO, neuroinflammation, sepsis

RESEARCH AREA: Biology

RESEARCH FIELD: Molecular neurobiology

REZIME

Smatra se da je neuroinflamacija jedan od zajedničkih imenitelja i ključnih faktora u procesu neurodegeneracije kod različitih poremećaja u funkcionisanju centralnog nervnog sistema (CNS), koji mogu biti izazvani traumatskom povredom mozga, moždanim udarom, neurodegenerativnim bolestima, sepsom, itd. Kod ovih patoloških stanja, pojavljuje se sličan obrazac dogadjaja, koji uključuje lokalnu sintezu medijatora inflamacije, otvaranje barijera CNS-a, ulazak leukocita, kao i aktivaciju ćelija mikroglije. Horoidni pleksus (HP) je struktura koja čini jednu od tri barijere CNS-a, krvno-likvornu barijeru (KLB). Nalazi se u sve četiri moždane komore i jedinstvene je strukture. Sastoji se iz epitelijalnih ćelija horoidnog pleksusa (EĆHP) spojenih čvrstim vezama koje okružuju fenestrirane kapilare. HP je odgovoran za većinsku proizvodnju cerebrospinalne tečnosti (CST), a na površini EĆHP postoje brojni transporteri i receptori, koji utiču na održavanje homeostaze i nesmetani rad mozga. Zbog svoje pozicije, strukture i funkcije HP je prepoznat kao ključni organ koji je uključen u nadzor imunskog odgovora u mozgu i kao važan faktor u komunikaciji između periferije i CNS-a u procesu inflamacije.

Glavni cilj ove teze je bio da se ispita doprinos HP inicijaciji neuroinflamacije, kroz promenu morfologije i funkcionalnosti EĆHP. U tu svrhu korišćena su dva mišija modela bolesti povezanih sa neuroinflamacijom: a) model sistemske inflamacije, lipopolisaharidom (LPS) indukovan model sepse i b) novouspostavljeni model Alchajmerove bolesti, indukovan intracerebroventrikularnom (i.c.v) aplikacijom oligomerima amiloida beta (AβO). Najvažniji rezultati ovog istraživanja pokazuju da je propusnost krvno-likvorne barijere (KLB) povećana kod AβO modela, a da je sam uzrok toga gubitak tipične kuboidne morfologije EĆHP, kao i smanjenje ekspresije komponenti čvrstih veza, kao i da je ova promena prolaznog karaktera i da se kroz nedelju dana barijera vraća na fiziološki nivo propustljivosti. Ispitivanje propustljivosti krvno-moždane barijere nije pokazalo nikakve značajne promene. Što se tiče promena funkcionalnosti HP, uočena je povećana ekspresija gena za ispitivane proinflamatorne citokine u HP i u hipokampusu, kao i njihov povišeni nivo u CST. Takođe, zabeleženo je povećanje ekspresije gena za određene matriksne metaloproteinaze (MMP) u HP, kao i njihova povišena aktivnost u CST. Nakon istovremenog injeciranja MMP inhibitora širokog spektra sa AβO, primećen je nivo propustljivost barijere kao kod intaktnih

životinja. Slično, smanjenje povećane propustljivosti KLB uzrokovane i.c.v. aplikovanjem AβO je uočeno i kod MMP3 deficijentnih miševa. Kada je ispitana aktivacija mikroglije, kao rezidentne imunske ćelije CNS-a, nije primećena značajna promena kod AβO modela.

Ustanovljeno je takodje i povećanje broja ekstracelularnih vezikula (EV) u CST, kao i povećanje ekspresije markera za EV, kao i mikroRNK-155, koja je prepoznata kao jedna od ključnih mikroRNK koje dovode do promene ekspresije gena uključenih u inflamatorni odgovor, a samim time i do povećanja odgovora na inflamatorni stimulus. Primećena je i prolaznost odgovora EĆHP kroz povećanje ekspresije EV i mikroRNK-155, nedelju dana nakon primene inflamatornog stimulusa. Sličan obrazac promena u HP nađen je i u modelu LPS-om indukovane sepse.

Dobijeni rezultati su ukazali na značajnu ulogu HP u inicijaciji neuroinflamacije, kroz strukturne i funkcionalne promene EĆHP, u dva različita modela bolesti povezanih sa neuroinflamacijom. Ovi rezultati ukazuju na HP kao potencijalni terapeutski target u poremećaju funkcionisanja CNS-a usled inflamatornih procesa.

KLJUČNE REČI: KLB, HP, MMP, EV, AβO, neuroinflamacija, sepsa

NAUČNA OBLAST: Biologija
UŽA NAUČNA OBLAST: Molekularna neurobiologija

SAMENVATTING

Neuroinflammatie wordt beschouwd als een veel voorkomende reden en cruciale speler in neurodegeneratie waargenomen bij verschillende aandoeningen van het centrale zenuwstelsel (CZS), zoals traumatisch hersenletsel, beroerte, neurodegeneratieve ziekten, sepsis, enz. Bij deze pathologische aandoeningen treedt een vergelijkbaar patroon van gebeurtenissen op, waaronder de lokale productie van inflammatoire mediatoren, het openen van de CZS barrière, transport van leukocyten en de activering van microgliale cellen. De plexus choroideus vormt één van de drie barrières van het CZS, meer specifiek de bloed-cerebrospinaal vocht barrière (BCSVB). De CP is een unieke structuur die zich bevindt in alle vier de hersenen ventrikelen, welke bestaat uit plexus choroideus epitheliale cellen (CPE), onderling verbonden via tight junctions die de gefenestreerde haarvaten omgeven. De CP produceert de meerderheid van het cerebrospinaal vocht (CSV), en talrijke transporters en receptoren zijn aanwezig op het oppervlak van de CPE cellen wat de homeostase van de hersenen beïnvloedt. Vanwege zijn positie, structuur en functie werd de CP de laatste tijd erkend als een belangrijke speler die betrokken is bij immuun surveillance in de hersenen, evenals een belangrijke factor in de communicatie tussen CNS en periferie tijdens het ontstekingsproces.

Het belangrijkste doel van dit proefschrift was om de bijdrage van de CP aan de initiatie van neuroinflammatie te onderzoeken. Hiervoor werden twee ziektemodellen geassocieerd met neuroinflammatie gebruikt: sepsis geïnduceerd via de systemische injectie van lipopolysaccharide (LPS) en een recent ontwikkeld model van de ziekte van Alzheimer geïnduceerd door intracerebroventriculaire (i.c.v) injectie van amyloïde beta-oligomeren (AβO). De belangrijkste bevindingen van deze studie tonen aan dat de permeabiliteit van de BCSFB verhoogd was in het AβO-model, gekenmerkt door het verlies van de typische kubusvormige morfologie van de CPE-cellen en door een verlaagde expressie van de tight junction componenten. Deze verandering bleken transiënt en herstelden zich terug binnen een week. Tegelijkertijd waren er geen significante veranderingen in de doorlaatbaarheid van de bloed-hersenbarrière.

In de CP werd een verhoogde genexpressie van verschillende cytokines waargenomen, evenals verhoogde eiwitlevels in het CSV. Daarnaast werd geen significante verandering waargenomen in de activering van microgliale cellen, residente immuuncellen in de hersenen, in het AβO model. De genexpressie van verschillende matrix metalloproteïnasen (MMP) in de CP was verhoogd en in het CSV werd een verhoogde MMP activiteit vastgesteld. I.c.v. injectie van een breedspectrum MMP remmer samen met AβO zorgde voor de preventie van de AβO-geïnduceerde BCSVB permeabiliteit. In overeenstemming hiermee werd een verminderde toename in BCSVB-permeabiliteit na AβO injectie waargenomen bij MMP3-deficiënte muizen. Verder werd een toename van het aantal deeltjes in de CSF en een toename in genexpressie van markers van extracellulaire vesicles (EV) en miR-155 gevonden in de CP van AβO geïnjecteerde muizen. Een vergelijkbaar patroon van veranderingen in de CP werd waargenomen als respons op LPS injectie in vergelijking met AβO injectie.

De resultaten van dit onderzoek onthulden een belangrijke rol van de CP bij de initiatie van neuroinflammatie, via structurele en functionele veranderingen, in twee verschillende modellen geassocieerd met neuroinflammatie. De verkregen resultaten wezen op de significante rol van CP bij de initiatie van neuroinflammatie, door structurele en functionele veranderingen in CPE-cellen, in twee verschillende modellen van ziekten geassocieerd met neuroinflammatie. Deze resultaten geven het belang aan van CP als een potentieel therapeutisch doelwit in de aandoeningen geassocieerd met neuroinflammatie.

SLEUTELWOORDEN: BCSFB, CP, MMP, EV, AβO, neuroinflammation, sepsis

ONDERZOEKGEBIED: Biologie

ONDERZOEKSVELD: Moleculaire neurobiologie

ABBREVIATIONS:

AA - amino acids

Aβ - amyloid beta

Aβ1-40 - 40 amino acids long amyloid beta

Aβ1-42 - 42 amino acids long amyloid beta

AβO - Aβ oligomers

AL - ad libitum

AD - Alzheimer's disease

APC - antigen presenting cells

APP -amyloid precursor protein

BBB - blood-brain barrier

BCSFB - blood-cerebrospinal fluid barrier

BDNF - brain derived neurotrophic factor

BSA - bovine serum albumin

BSF-2 - B-cell stimulatory factor-2

CNS - central nervous system

CSF - cerebrospinal fluid

CSFs - colony-stimulating factors

CPE - choroid plexus epithelial

CTL - Cytotoxic T lymphocytes

DAMPs - danger-associated molecular patterns

DNA - deoxyribonucleic acid

ECF - extracellular fluid

ECM - extracellular matrix

EVs - extracellular vesicles

FGF - fibroblast growth factor

G-CSF - granulocyte colony-stimulating factor

GM-CSF - granulocyte-macrophage colony-stimulating factor

HFIP - hexafluoroisopropanol

Iba-1 – ionized calcium binding adaptor molecule 1

ICAM-1 - intercellular Adhesion Molecule 1

i.c.v. – intracerebroventricullarly

IGF - insulin growth factor

IFNs - interferons

ILs - interleukins

ILVs - intraluminal vesicles

i.p. - intraperitoneal

i.v. - intravenously

JAMs - junction adhesion molecules

LPS - lipopolysaccharide

MAGUK - membrane-associated guanylate kinase

MCP-1 - Macrophage chemotactic protein-1

MHC - major histocompatibility complex

MIP-1α - macrophage inflammatory protein-1

miRs - MicroRNAs

MMPs - Matrix metalloproteinases

MODS -multiple organ dysfunction

mRNAs - messenger RNAs

MS - Multiple sclerosis

MVEs - multivesicular endosomes

NGF - nerve growth factor

NFTs -neurofibrillary tangles

NK - Natural killer

NO - nitric oxide

NT - neurotrophins

NTA - Nanoparticle tracking analysis

PAMPs - pathogen-associated molecular patterns

PBS - phosphate-buffered saline

PCR - Polymerase Chain Reaction

PDGF - platelet derived growth factor

PRRs - PAMPs are recognized by pattern recognition receptors

PS1 - presenilin 1

PS2 - presenilin 2

RANTES - regulated on activation, normal T-cell expressed and secreted

ROS - reactive oxygen species

RT - room temperature

SAE - sepsis associated encephalopathy

SEM - standard error of mean

SIRS - systemic inflammatory response syndrome

SPF - specific pathogen-free

TACE - TNF converting enzyme

TBI - traumatic brain injury

TGF - transforming growth factor

TGF-β - transforming growth factor β

Th - T helper cells

TIMP - tissue inhibitor of metalloproteinases

TLR - Toll like receptor

tmTNF - transmembrane precursor protein of tumor necrosis factor

TNF - tumor necrosis factor

TNFR - tumor necrosis factor receptor

TTR - transthyretin

VCAM-1 -vascular cell adhesion protein 1

VEGF - vascular endothelial growth factor

ZO - zonula occludens

Table of contents

1. Introduction

1.1. Central nervous system (CNS) barriers

Since neuronal networks demand stable extracellular fluid (ECF) even subtle changes in the microenvironment of the brain are enough to cause a detrimental effect on the central nervous system (CNS) function,. CNS homeostasis is maintained by the presence of the skull, spine and several brain barriers, including the arachnoid or meningeal barrier, the blood-brain barrier (BBB), and the blood-cerebrospinal fluid barrier (BCSFB), as well as spinal cord barrier and the blood-retinal barrier (***Figure 1***). These barriers separate the CNS from the periphery, and ensure that blood fluctuations do not affect brain functioning and sustain ECF specialised composition (De Bock et al., 2014). Beneath the skeletal parts there are two meningeal layers, called the dura and leptomeninges. Leptomeninges are comprised of **arachnoid mater** and pia mater, building the perivascular or Virchow-Robin space. Arachnoid meninges are composed of cells interconnected with tight junctions (TJs) and surrounded by continuous basal lamina on its inner surface, forming arachnoid barrier (Abbott et al., 2010). Recently, the existence of a **glymphatic system** in the brain was confirmed. It was shown that dural lymphatic vessels take up cerebrospinal fluid (CSF) from the subarachnoid space and brain interstitial fluid, further transporting it into deep cervical nodes (Aspelund et al., 2015). Both T and B cells were reported to be present in the dural lymphatic vessels (Louveau et al., 2015). However, peripheral immune cells are largely prevented to enter the CNS due to the presence of the different CNS barriers. The **BBB** is comprised of a neurovascular unit, containing endothelial cells interconnected with TJs forming tight walls of the brain capillaries. These endothelial cells differ from other endothelial cells in the body, since they lack fenestrations and have firmer TJs that restrict paracellular transport from blood to the brain, limiting the transport to be mainly transcellular. Endothelial cells of the BBB express highly specific transporters, therefore allowing selective entry of the molecules into the brain (Abbott et al., 2010). Besides the endothelial cells, the neurovascular unit also consists of pericytes that lie on top of the endothelial cells, regulate capillary flow and BBB permeability

(Armulik et al., 2010). Endothelial cells and pericytes are surrounded by basal lamina, on which astrocyte end-feet are laid upon (Bonkowski et al., 2011). The focus of this dissertation is the **BCSFB** located at the choroid plexus (CP), a largely understudied part in the brain, which will be discussed in detail below (1.1.1).

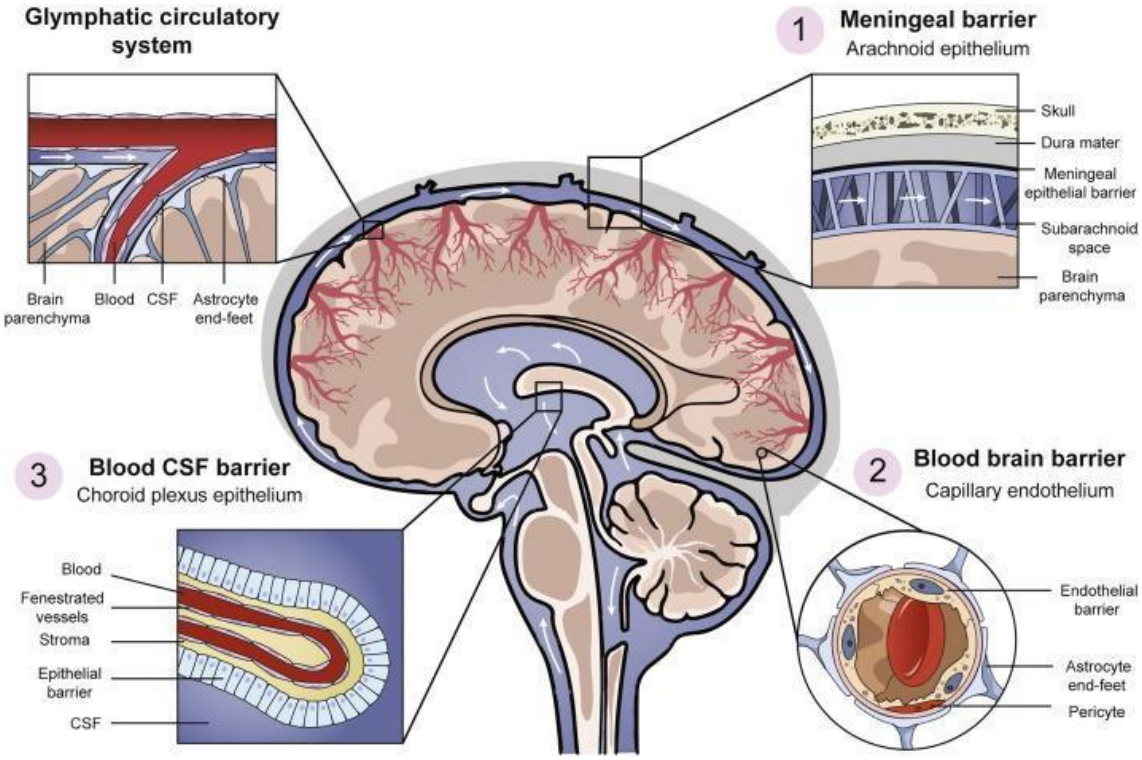

Figure 1. Schematic representations of three brain barriers and brain glymphatic circulatory system. *In the CNS three barriers can be found: 1. Meningeal barrier; 2. Blood brain barrier; and 3. Blood-CSF barrier. Lately, presence of glymphatic system, has been shown in the brain (adapted from Stock et al., 2017).*

1.1.1. Blood-cerebrospinal fluid barrier (BCSFB)

The primary role of the CP is CSF production, through passive filtration of plasma through fenestrated endothelium. CSF has several roles in the brain: it serves as a protection from trauma or alternations in blood pressure, facilitates

transport of various molecules throughout CNS, and removes toxins and metabolic by-products (Balusu et al., 2016a). The regulation of the composition of CSF is very important, since every fluctuation will affect the extracellular environment of astrocytes and neurons surrounding the ventricles (Sakka et al., 2011). Interestingly, CSF in humans is replaced 3 to 4 times per day (Kaur et al., 2016).

The BCSFB is present in all four brain ventricles and is formed by a single layer of CP epithelial (CPE) cells interconnected by TJs and surrounding a vascular network. On the basal side, CP is separated from the brain parenchyma by ependymal cells lining the ventricles, and at the apical side of the CPE cells macrophages named epiplexus cells could be observed. Between fenestrated endothelial cells and CPE cells is an area called stroma where resident inflammatory cells can be found, such as antigen presenting cells (APC) and resident macrophages (***Figure 2***).

During embryonic development, CPE cells develop from the roof plate neuroepithelium (Hunter and Dymecki, 2007). The CP presents a highly vascularised structure, where fenestrations help filtration of blood for CSF production. As a consequence, blood flow level is 10 times larger in this area than in the rest of the brain, enabling CPE cells to produce more than two thirds of CSF in the brain (Damkier et al., 2013; Szmydynger-Chodobska et al., 1994).

1.1.2. CPE cell morphology and barrier function

CPE cells are cuboidal in shape with a large centrally positioned nucleus. At the basal side there are numerous invaginations of the membrane called the basolateral labyrinth, while at the apical side numerous microvilli exist. Both characteristics greatly expand the surface area available for transport between stroma and CSF. To be able to effectively regulate paracellular transport from blood, and thus the composition of CSF, CPE cells are interconnected with junctions at their apical lateral side. Their existence prevents entrance of large, hydrophilic,

highly polarized molecules, and allows passage of only small lipophilic molecules, such as O_2 and CO_2 (Gorle et al., 2016). These junctional complexes between CPE cells are formed by several types of junctions: TJs, adherens junctions, gap junctions and desmosomes. The general morphology of TJs at the BCSFB is similar to TJs at the BBB (Redzic, 2011). TJs present on the apical side of the cell are comprised of three integral membrane proteins: claudins, occludins and junction adhesion molecules (JAMs). They are supported by cytoplasmic accessory proteins, such as members of zonula occludens (ZO) protein family, which link them to actin cytoskeleton. Occludin is a 60 kDa integral membrane protein and claudins are a family of 20-34 kDa membrane proteins (Lal-Nag and Morin, 2009). TJ stability is assured by a network of cytoplasmic proteins, such as membrane-associated guanylate kinase (MAGUK) family members, among which some of the most important are ZO-1, -2 and -3. ZO-1 forms heterodimers with ZO-2 and ZO-3 and together they interact with both claudins and occludins. It has been shown that ZO proteins are crucial for the assembly of claudins and occludins at TJs (Saitou et al., 1998; Umeda et al., 2006).

Adherens junctions are present below TJs and are formed by cadherins and associated proteins that bind them finally to actin cytoskeleton. Cadherins consist of a large family of transmembrane proteins that can be classified as classical, desmosomal, protocadherins and unconventional cadherins. E- and N-cadherin are the most studied classical cadherins (Saito et al., 2012).

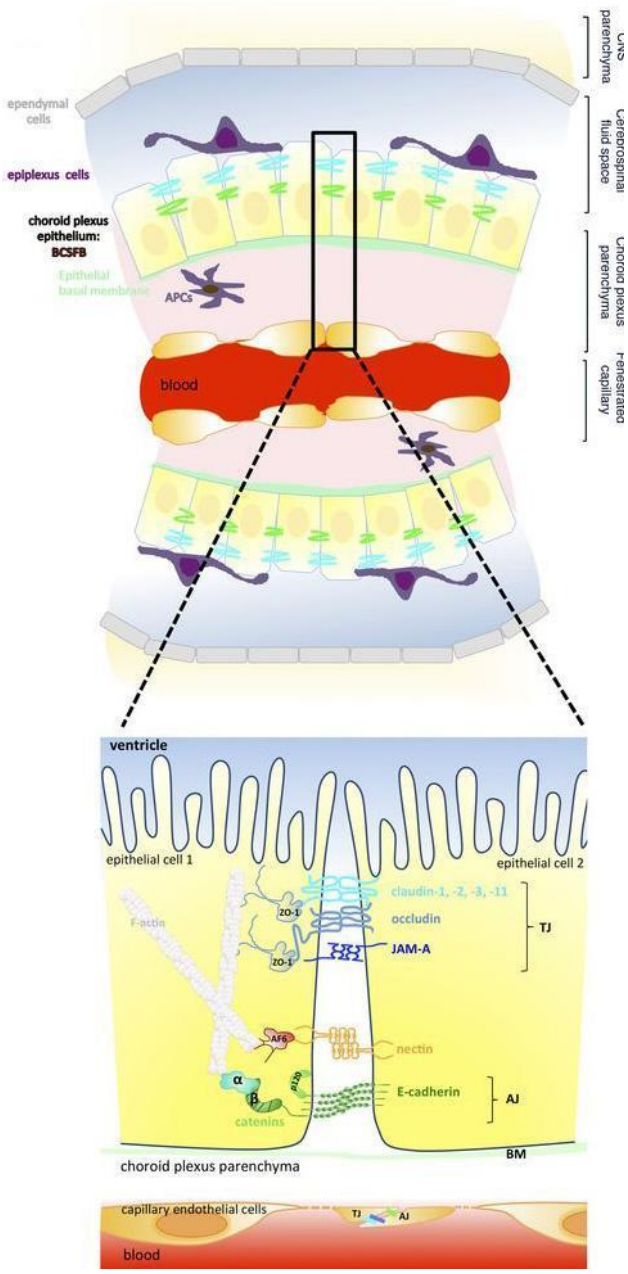

Figure 2. ***Structure of the BCSFB and junctional complexes between CPE cells.*** *The BCSFB is formed by CPE cells connected with tight junctions (TJs). On epithelial cells, epiplexus cells could be found, while in the choroid plexus (CP) stroma, among others, antigen presenting cells (APCs) reside. Between CPE cells, besides TJs, also adherens and gap junction exist (adapted from Tietz and Engelhardt, 2015).*

The cytoplasm of the CPE cells is densely populated with mitochondria (15-20% of volume) Golgi apparatus and endoplasmic reticulum, enabling CPE to be an active site of peptide and protein synthesis and secretion (Cornford et al., 1997). After being secreted into the CSF, these molecules flow through the ventriculo-subarachnoid space to specific neuronal and glial targets in brain parenchyma.

CPE cells also contain high levels of glutathione, cysteine and metallothioneins that can harbor neurotoxic molecules from the CSF. Additionally, CPE cells also contain enzymes such as superoxide-dismutase and glutathione peroxidase, that play an active role in protection against oxidative stress.

1.1.3. Secretory activity of the CP tissue

Transport occurring between brain and periphery through brain barriers is highly regulated and revolving through specific transporters/receptors. CPE cells express numerous transporters and receptors on the apical and basolateral side. Some of them are involved in the CSF production and secretion, while others help surveil and respond to biochemical changes in the brain. Transport is facilitated for various nutrients, hormones, polypeptides, as well as restricted for many molecules, through synergism of TJs, active transport and metabolic enzymes. Glucose transporters, amino acids and nucleoside transporters, as well as various peptides transporters are present. Additionally, ion transporters and channels for Na^+, K^+, Cl^-, $HCO3^-$, Ca^{2+} and Mg^{2+} are present on the CPE membrane and participate in CSF formation (***Figure 3***) (Spector et al., 2015).

In physiological conditions, the CP functions as an autocrine, paracrine and endocrine organ in the brain. It is the production site of transthyretin (TTR) or prealbumin that is secreted into the CSF and acts as a carrier protein for thyroxine hormone (T4) secreted by thyroid gland. mRNA levels and activity of this hormone are strictly maintained stable in CNS (Burmeister et al., 1997). Although it has been known that TTR regulates brain growth in the development, and that its potential

role is in sequestering thyroid hormones in the brain, the exact role of TTR in the adult brain remains elusive (Chodobski and Szmydynger-Chodobska, 2001). However, it was shown that it is not necessary for T4 entry in the brain (Dratman et al., 1991). Moreover, CPE cells in the adult brain secrete various growth and transcription factors. Brain derived neurotrophic factor (BDNF), nerve growth factor (NGF) and neurotrophins (NT) are highly secreted by the CPE cells during development and to lesser extent in the adult brain, helping them in proliferation, survival and differentiation of neurons (Karen Arnaud, 2016). Insulin growth factor (IGF), fibroblast growth factor (FGF), transforming growth factor β (TGF-β), platelet derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) are also produced by the CPE cells and promote angiogenesis, proliferation, plasticity and cognition (Marques et al., 2011). Additionally, also in inflammatory processes, the CP is a site of secretion of various biologically active molecules (Mietelska-Porowska and Wojda, 2017; Strazielle et al., 2003).

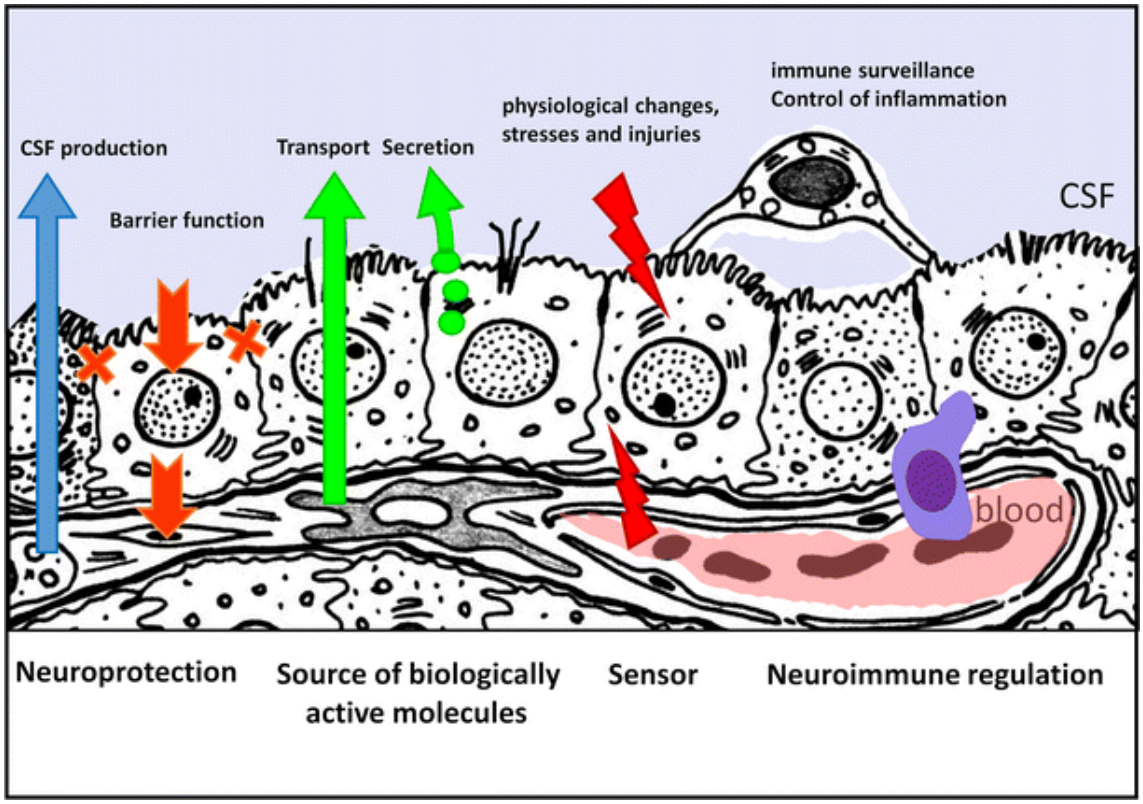

Figure 3. Schematic representation of the main choroid plexus (CP) functions. *The CP, besides its barrier function, is involved in CSF production, transport and secretion of various biologically active molecules, thus being able to respond to physiological and pathological changes, and inflammatory response. (adapted from Ghersi-Egea et al., 2018)*

1.2. Inflammation

The inflammatory response is a protective, complex biological and biochemical response of the living tissue, elicited by injury or infectious agents, involving biological mediators and cells of the immune system (Medzhitov, 2008). The immune system consists of the innate general defense system and the acquired adaptive immune system. The innate immune system acts as a first line of defense that recruits other immune cells to the site of infection or insult through production of cytokines. Several types of white blood cells are involved in the

innate immune system. Firstly, monocytes, which after migrating to tissue become macrophages, and phagocyte debris and pathogens. Secondly, granulocytes, which contain granules in their cytoplasm, that are used to destroy microbes. Three types of granulocytes exist of which most numerous are neutrophils (that have phagocytic activity), eosinophils, basophils and mast cells. Dendritic cells (DCs) also play a role in innate immunity via phagocytosis and antigen presentation. One type of T cells, called Natural killer (NK) cells, are part of innate immunity, and they recognize cells that lack self-major histocompatibility complex (MHC) and destroy them. Interestingly, all immune cells are derived from pluripotent progenitor cells in the bone marrow, which, through stimulation with different cytokines and other signals, mature to different cell types. Besides immune cells, the complement system is also a component of innate immunity, and aids antibodies and phagocytic cells in removing pathogens and damaged cells.

The innate immune system activates the adaptive defense, which is specific pathogen targeted immunity. Adaptive immune response consists of T and B lymphocytes, in addition to the antibodies produced by B lymphocytes (Janeway CA Jr et al., 2001). T cells express specialized receptors that recognize antigens on their surface. T helper (Th) cells, or CD4+ T cells have function to communicate with other cells thus coordinating immune response. They can activate B cells to produce antibodies, and activate more T cells or phagocytes. On the other hand Cytotoxic T lymphocytes (CTL), attack cells that carry foreign antigen presented on self MHC.

Acute inflammation is characterized by transient episodes of inflammatory response. It could be described as a nonspecific response of the body to an insult, lasting seconds, minutes, hours or days, that is elicited by the resident immune cells within the affected tissue. This type of inflammation mostly involves an increased blood flow and permeability of the capillaries allowing penetration of blood components, especially leukocytes, into interstitial spaces. During acute inflammation, neutrophils are the predominant type of immune cells present (Rankin, 2004).

When the acute inflammation is prolonged, it enters the **chronic** phase. Besides the continued increase in blood flow and permeability of the capillaries, macrophages and lymphocytes also migrate from blood to tissue. Macrophages play a main role in the phagocytosis of pathogens. Additionally, they secrete a range of inflammatory mediators and growth factors. T cells are also present at the site of injury/inflammation with the role to activate macrophages and B cells to produce antibodies locally (Murakami and Hirano, 2012).

In the case of pathogen infection, inflammatory response is initiated by pathogen-associated molecular patterns (PAMPs) (Kang et al., 2015). PAMPs are molecules conserved within the class of microbes (*e.g.* lipopolysaccharide (LPS) - endotoxin present in the cell membrane of gram-negative bacteria). In the host organism, PAMPs are recognized by pattern recognition receptors (PRRs), a class of receptors present on the surface of innate immune cells (*e.g.* Toll like receptor-4 (TLR) for LPS). TLRs are shown to be the key players in pathogen recognition. Interaction of PAMPs and PRRs initiates an intracellular cascade that leads to activation of a proinflammatory response. Moreover, PAMPs, along with other stress stimuli can induce secretion of danger-associated molecular patterns (DAMPs) from immune cells, that can also bind to PRRs and stimulate release of inflammatory mediators, thus contributing to the maintenance of chronic, uncontrolled inflammation (Kang et al., 2015).

The inflammatory process is strictly controlled by a plethora of extracellular mediators and regulators. Cytokines, growth factors, eicosanoids, complement and peptides are all included in the complex regulation of inflammation through their ability to induce various intracellular signaling pathways on different cell of both innate and adaptive immune system (Lin and Karin, 2007).

1.2.1. Inflammatory mediators

1.2.1.1. Cytokines

The term cytokine is a common name for all interleukins (ILs), chemokines, colony-stimulating factors (CSFs), interferons (IFNs), transforming growth factors (TGF) and tumor necrosis factor (TNF) families. Cytokines are small proteins, serve in the intercellular communication in picomolar or nanomolar concentration, and represent one of the key players in inflammatory response (Ramesh et al., 2013). They can be produced by both immune and non-immune cells and can exert either inhibitory (anti-inflammatory) or stimulatory (proinflammatory) effects on the inflammatory process. Cytokines show several characteristics. Firstly, they act synergistically. Secondly, they act redundantly (different cytokines, same effect), and pleiotropically (one cytokine different effects on different cells). Thirdly, they can have autocrine or paracrine activity, and in a small number of cases also endocrine function (Zhang and An, 2007). They are able to convey their functions through binding to the cell membrane receptors, and initiating signaling pathways that finally lead to the upregulation or downregulation of the transcription of cytokine-regulated genes.

Regarding their biological function in the context of inflammatory response most cytokines can be differentiated into four major groups: (1) innate immunity (*e.g.* IL-1, -5, -6, and -8); (2) management of inflammatory processes (*e.g.* IL-1, and -4, and TGF-β); (3) lymphocyte activation and proliferation (*e.g.* IL-2 and -4); and (4) leukocyte growth mediation (*e.g.* IL-1, -3, -5, and -6) (Dahm et al., 2016).

Immune responses, especially trafficking and maturation of immune cells, are controlled through production and secretion of cytokines and chemokines by various cells. Thus, a specific cytokine profile determines host defense and leads to leukocyte trafficking to CNS (Dahm et al., 2016). In this thesis several important cytokines for the inflammatory processes will be further discussed (***Table 1***).

1.2.1.1.1. Interleukines (IL)

The two mostly studied members of the IL1 family are IL-1α and IL-1β. Both are synthesized as a proform, that is cleaved into the mature form. Moreover, both bind to the IL1R1 cell surface receptor (Di Paolo and Shayakhmetov, 2016). IL-1α was shown to be a proinflammatory, pyrogenic cytokine, activated early in inflammation. In stimulated state, IL1α extends lifespan of neutrophils and macrophages and promotes differentiation of lymphoid cells (Di Paolo and Shayakhmetov, 2016). IL-1β, the most investigated interleukin, is a potent proinflammatory cytokine. It has stimulatory effect on lymphocytes and promotes their differentiation (Santarlasci et al., 2013).

Other important IL superfamily members include IL-3; a cytokine with a double role. On one hand it is responsible for stimulation of bone marrow progenitor cells to proliferate and differentiate into different blood cells. On the other hand, its ability to activate monocytes, basophils and eosinophils in inflammatory processes has been shown (Korpelainen et al., 1996). Next, IL-6 is an important cytokine with pleiotropic effects (Jucker et al., 1991). IL-6, also called B cell stimulatory factor-2 (BSF-2), is a key player in differentiation of B cells into immunoglobulin-secreting cells. It also has a role in recruitment and anti-apoptosis of T lymphocytes (Scheller et al., 2011). IL-6 is secreted in initial phases of inflammation locally, following which it acts as warning signal to the rest of the body (Tanaka et al., 2014). IL-6 stimulates activated B cells to secrete antibodies, T cells and various other cells in the organism. In contrast, IL-10 is a potent anti-inflammatory cytokine that plays a central role in limiting host immune response, thereby preventing damage to the host and maintaining normal tissue homeostasis (Iyer and Cheng, 2012). IL-10 mostly downregulates expression of certain cytokines (de Waal Malefyt et al., 1991). IL-12p40 is a subunit that plays a role in attracting macrophages and DCs toward the site of inflammation and is able to induce IFN-γ secretion (Cooper and Khader, 2007). Two additional important members of the IL superfamily are IL-13 and -17A. IL-13 is a cytokine with pleiotropic effects which mostly downregulates expression of proinflammatory

cytokines such as TNF and IL-6. It also acts on monocyte and lymphocyte proliferation and differentiation (Seyfizadeh et al., 2015). IL-17A is able to stimulate production of other cytokines, chemokines and matrix metalloproteinases (MMPs) (Gaffen, 2008).

1.2.1.1.2. Chemokines

Chemokines or chemoattractant cytokines, are involved in attraction or movement of the cells, and are produced immediately after the injury/infection. They are small molecules, of a size between 8-14 kDa. Usually they are produced by leukocytes or endothelial cells. There are four subgroups of chemokines, C-C, C-X-C, X-C and CX3C, divided by the presence of a residue between the first two cysteines ("X" represents any amino acid) (Allen et al., 2007). C-C chemokines attract monocytes, basophils, eosinophils, and lymphocytes, while the majority of C-X-C chemokines attract neutrophils. An important member of the chemokine superfamily is 'regulated on activation, normal T cell expressed and secreted' (RANTES) or C-C chemokine Ligand 5 (CCL5) (Marques et al., 2013b). RANTES exerts its effects on T cells, DCs, eosinophils, NK cells, mast cells and basophils (Marques et al., 2013b). Macrophage chemotactic protein-1 (MCP-1) or CCL2 is expressed by neurons, astrocytes, microglia, CPE cells, and its role is to attract monocytes/macrophages (Deshmane et al., 2009). Other important chemokines involved in the inflammatory process are macrophage inflammatory protein-1 (MIP-1α) or CCL3 and MIP-1β or CCL4, which are both involved in granulocytes activation leading to the induction of proinflammatory cytokines such as IL-1, IL-6 and TNF, and chemotaxis of monocytes (Schwerk et al., 2015).

1.2.1.1.2. Colony-stimulating factors (CSFs)

Granulocyte colony-stimulating factor (G-CSF) leads to an increase in the number of neutrophils (Bendall and Bradstock, 2014). Another member of this cytokine group is granulocyte-macrophage colony-stimulating factor (GM-CSF) or colony stimulating factor 2 (CSF2), which has a role to stimulate granulocytes, and induce growth and differentiation of macrophages/monocytes and DCs (Becher et al., 2016).

1.2.1.1.3. Tumor necrosis factor (TNF) superfamily

Tumor necrosis factor (TNF) is the most studied member of tumor necrosis factor superfamily type II transmembrane proteins. It plays an important role in cell death, cell proliferation and differentiation. TNF is a key molecule in the inflammatory activity of the innate immune system, being involved in cytokine production and in activation and expression of adhesion molecules. It has been shown that besides CPE cells, monocytes, macrophages, T cells, B cells, neutrophils, NK cells and endothelial cells can also secrete TNF. Its endocrine, as well as paracrine function on lymphocytes, macrophages and neutrophils has been identified. Firstly, TNF is synthesised as a transmembrane precursor protein (tmTNF). Afterwards it associates into non-covalent trimers in the plasma membrane, and then is being cleaved by the metalloprotease, TNF converting enzyme (TACE or ADAM17) (Black et al., 1997). TNF binds to either tumor necrosis factor receptor 1 (TNFR1) or TNFR2 (Tracey et al., 2008). However, proinflammatory effects of TNF appear to be mediated predominantly through TNFR1 (Probert et al., 2000). Binding to the receptor, TNF leads to kinase cascades that trigger proinflammatory gene expression.

1.2.1.1.4. Interferons (IFNs)

The best known interferon (IFN) is INF-γ, which belongs to type II interferons, and it is known to be activated by IL-12. INF-γ is leading to induction of cell-mediated immune responses through JAK/STAT signaling pathway (Mitagami et al., 2015). INF-γ has an effect on immune, as well as the other cells types, and in macrophages, it induces cytokine and NO production, thus aggravating inflammation. INF-γ is shown to be the key molecule in T cells trafficking across CP tissue (Kunis et al., 2013).

Table 1. Important cytokines involved in inflammatory processes and their function. *Cytokines are classified in several major classes, such as interleukins, chemokines, colony stimulating factors, tumor necrosis factor and interferons. These biologically active molecules have important functions in inflammatory processes.*

CYTOKINES	
Cytokine class	**Function**
Interleukines	
IL-1α	Extends lifespan of neutrophils and macrophages and promotes differentiation of lymphoid cells
IL-1β	Stimulates lymphocytes and promotes their differentiation
IL-3	Stimulates bone marrow progenitor cells and activates monocytes, basophils and eosinophils
IL-6	Stimulates T cells, activated B cells to secrete antibodies, and various other cells in the body

IL-10	Downregulates expression of other cytokines
IL-12p40	Attracts macrophages and DCs and induces IFN-γ secretion
IL-13	Downregulates expression of proinflammatory cytokines, such as TNF and IL-6
IL-17A	Stimulates production of other cytokines, chemokines and MMPs
Chemokines	
RANTES	Stimulates T cells, DCs, eosinophils, NK cells, mast cells and basophils
MCP-1	Attracts monocytes/macrophages
MIP-1α	Induces chemotaxis
MIP-1β	Induces chemotaxis
Colony stimulating factors	
GM-CSF	Stimulates granulocytes, induces growth and differentiation of macrophages/monocytes and DCs
Tumor necrosis family	
TNF	Stimulates cytokine production and activation and expression of adhesion molecules
Interferons	
INF-γ	Induces cytokine and NO production

1.2.1.2. Matrix metalloproteinases (MMPs)

MMPs are calcium (Ca^{2+}) dependent zinc (Zn^{2+}) containing endopeptidases produced as zymogens (pro-MMP), that have to be activated by other enzymes or free radicals, in order to exert their effects. They play numerous important roles in both physiological and pathological processes. In immune processes they cleave various molecules, such as growth factors, death receptors, chemokines and cytokines (Cauwe et al., 2007), leading to either pro- or anti-inflammatory effects (Le et al., 2007). MMPs are often upregulated by several cytokines and some MMPs can activate TNF and TGF-β, while other MMPs are able to degrade IL-1β (Ito et al., 1996; Vandenbroucke et al., 2013; Yu and Stamenkovic, 2000).

MMPs are multidomain proteins, and can contain N-terminal signal peptide (cleaved in the secretory pathway), propeptide (responsible for the latency), catalytic domain, hinge region and C-terminal hemopexin-like domain (react with substrate and tissue inhibitor of metalloproteinases (TIMP)) (Visse and Nagase, 2003). In general, MMPs can be classified into four subgroups: (1) gelatinases (MMP-2, MMP-9), (2) matrilysins (MMP-7, MMP-26), (3) archetypal MMPs, and (4) furin-activated MMPs (***Figure 4***).

Members of the archetypal MMPs are: stromelysins (MMP-3 and -10), collagenases (MMP-1, -8, and - 13) and other MMPs (MMP-12, -18, -20 and -27). In the subgroup of furin-activated MMPs belong secreted MMPs (MMP-11, -21, and -28), type-I transmembrane MMPs (MMP-14, MT1-MMP, MMP-15, MT2-MMP, MMP-16 (MT3-MMP), and MMP-24 (MT5- MMP)), type-II transmembrane MMPs (MMP-23), and GPI-anchored MMPs (MMP-17 (MT4-MMP) and MMP-25 (MT6-MMP)).

Because of their activity, MMPs have to be precisely regulated. Thus, they can be modulated on four levels: transcriptional activation, removal of the prodomain, interaction with components of extracellular matrix (ECM) and inhibition by endogenous inhibitors such as TIMPs (Loffek et al., 2011). Four TIMPs, TIMP1, -2, -3 and -4, regulate MMP activity. TIMPs are proteins of 21–28

kDa which bind the active site of MMPs in a one-to-one ratio (Nagase and Woessner, 1999). Additionally, α2 macroglobulin and receptor mediated endocytosis can inhibit activated MMPs.

MMPs are actively secreted by various types of cells and this process can be activated by proinflammatory cytokines (e.g., TNF and IL-1β) and several growth factors can initiate an intracellular signaling cascade leading to the activation of transcription factors, resulting in MMP transcription (Deschamps and Spinale, 2006). From MMP-zymogen state, mostly serine proteases or other MMPs initiate proteolytic cleavage of the prodomain; for example, MMP-3 can activate MMP-9 (Vempati et al., 2007). Also, high levels of reactive oxygen species (ROS) and reactive nitrogen species can induce activation of MMPs (Okamoto et al., 2001). After activation, MMPs mostly exert their biological function on components of the extracellular matrix, junctional complexes and basal lamina, thus liberating passage from blood to the brain through the barriers opening (Brkic et al., 2015a). Additionally, they are shown to play a role in proinflammatory cytokines and free radicals activation (Candelario-Jalil et al., 2009).

On the other hand, MMPs can have a beneficial effect on the tissue. They are involved in the degradation of protein aggregates (Taniguchi et al., 2017), as well as in the degradation of proinflammatory cytokines, such as IL-1β (Manicone and McGuire, 2008).

Especially, MMPs have been implicated in perpetuation of chronic inflammation, in peripheral chronic diseases, such as atherosclerosis, but also in neuroinflammatory components of the brain diseases, such as stroke, ischemia and bacterial meningitis (Brkic et al., 2015a; Candelario-Jalil et al., 2009; Newby, 2005). Also, MMPs are mentioned as possible biomarkers for different neuroinflammatory states, since they are mostly not detectable in the brain in physiological conditions (Simats et al., 2016).

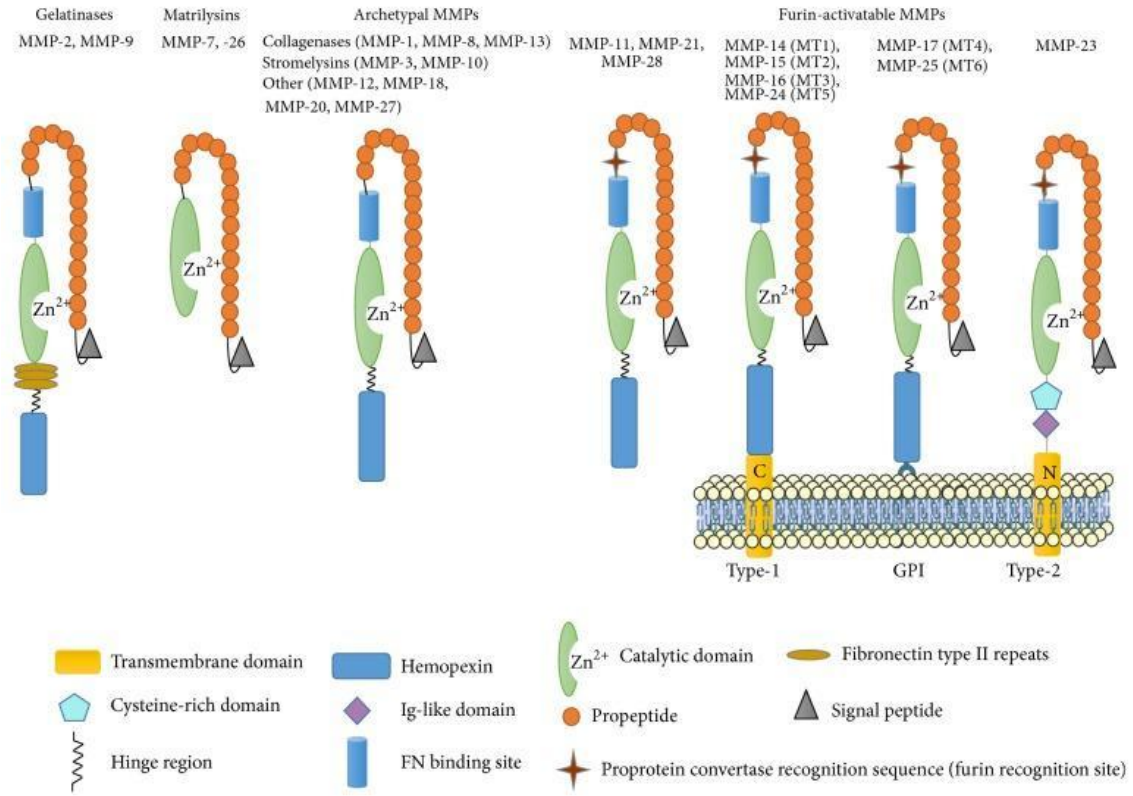

Figure 4. Classification of the MMPs based on their domain organization. *Four major groups of MMPs exist: gelatinases, matrilysins, archetypal MMPs, and furin-activatable MMPs. The typical structure of MMPs consists of a signal peptide, propeptide, a catalytic domain, hinge region, and a hemopexin domain. In addition, members of the gelatinases family have extra fibronectin type II motif repeats in the catalytic domain, and matrilysins have neither a hinge region nor hemopexin domains. Furin-activatable MMPs contain a furin recognition motif and are subcategorized into either secreted or membrane bound. Based on the type of membrane attachment, they are subdivided into type I transmembrane MMPs, GPI-linked MMPs, and type II transmembrane MMPs. Type-II transmembrane MMPs lack a cysteine switch. Instead, they have a cysteine rich domain and IgG-like domain. Abbreviations: C, C-terminal domain; FN, fibronectin; GPI, glycophosphatidylinositol; MMP, matrix metalloproteinases; N, N-terminal domain (adapted from Brkic et al., 2015a).*

1.2.1.3. Extracellular vesicles (EVs)

Due to their ability to carry nucleic acids, lipids and proteins, extracellular vesicles (EVs) are recognized as cell-to-cell communication mediators (Buzas et al., 2014). They play an important role in numerous physiological processes, including CNS development. On the other hand they are involved in pathological processes, such as neurodegenerative and inflammatory conditions in the CNS (Gupta and Pulliam, 2014). For example, they are involved in the processing of misfolded/aggregation-prone proteins in the CNS diseases (Rajendran et al., 2006). Moreover, they act on immune regulation in the brain and the rest of the body (Cossetti et al., 2012).

Every cell is shown to be capable of producing EVs that is an evolutionary conserved process (Deatherage and Cookson, 2012). Interestingly, gram-negative bacteria are found to be able to secrete EVs, called outer membrane vesicles (OMVs) that carry antigens and PAMPs such as lipopolysaccharide (LPS) (Buzas et al., 2014). Moreover, it has been shown that stressed or injured tissue secretes EVs containing DAMPs, such as heat-shock proteins, that have a role in initiation and propagation of inflammation, through binding to TLRs (Goh and Midwood, 2012). Also, CPE cells were shown to secrete EVs directly into the CSF in physiological and pathological conditions (Grapp et al., 2013; Tietje et al., 2014).

EVs can be classified into different categories, and classification method based by size (van Niel et al., 2018) identifies microvesicles (50–500 nm, even up to 1μm) and exosomes (50-150 nm). Difference between microvesicles and exosomes is that microvesicles present large membranous vesicles, produced by plasma membrane budding, while exosomes are intraluminal vesicles (ILVs) generated through invagination inside the endosomes during their maturation to multivesicular bodies (MVBs), and are only secreted after fusion of MVBs with cell membrane. Microvesicles cannot be easily distinguished from exosomes, due to the similar form, size and often composition. Mostly, circulating vesicles include both,

exosomes and microvesicles, and the same cells have been found to produce them simultaneously (***Figure 5***) (Raposo and Stoorvogel, 2013).

EVs can act by directly binding to ligands present on the cell surface followed by merging with the membrane and releasing their luminal content into the cytoplasm, or entering into cells *via* endocytosis, and acting inside the cell by altering its physiological environment. EVs are highly enriched in tetraspanins, which are a superfamily of proteins that are found in membrane microdomains called tetraspanin-enriched microdomains. They are interacting with various signaling proteins, with the role of promoting vesicular fusion or fission (Andreu and Yáñez-Mó, 2014). Some common markers for both, microvesicles and exosomes, are tetraspanins CD9, CD81, and CD63. Moreover, CD63 is one of the most frequently identified protein in EVs (Andreu and Yáñez-Mó, 2014).

The type of carried cargo is dependent on donor cell, as well as on physiological and pathological conditions that induced their production and secretion. Of specific interest are nucleic acids that can have a regulatory role in target cells. Interestingly, some messenger RNAs (mRNAs) are readily translatable in the recipient cell, but the majority of them are mostly regulatory (Batagov and Kurochkin, 2013). Also, microRNAs (miRs), which are small (from 21-23 nucleotides in length), non-coding RNAs, are able to regulate gene expression on a post-transcriptional level through inhibition of translation or destabilisation of mRNAs (Bartel, 2009). MicroRNAs (miRs) can be found in CSF, blood, plasma, urine, saliva and sperm, and are regarded as putative biomarkers in different diseases (Benz et al., 2016; Galimberti et al., 2014). It has been long known that miRs play an important role in immune regulation. However, recently it has been discovered that EVs carry miRs to be able to exert such activity, by dysregulation of gene expression in target cell (O'Connell et al., 2010). Interestingly, miRNAs can regulate TLR signalling, which further triggers Nf-kB pathway, and leads to production of proinflammatory cytokines (Fabbri, 2012). Especially, miR-155 was shown to play important role in inflammatory processes, and to influence alternations in BBB functioning. Moreover, EVs are shown to carry

proinflammatory cytokines, such as IL-1β (Nickel and Rabouille, 2009), as well as MMPs (Shimoda and Khokha, 2013).

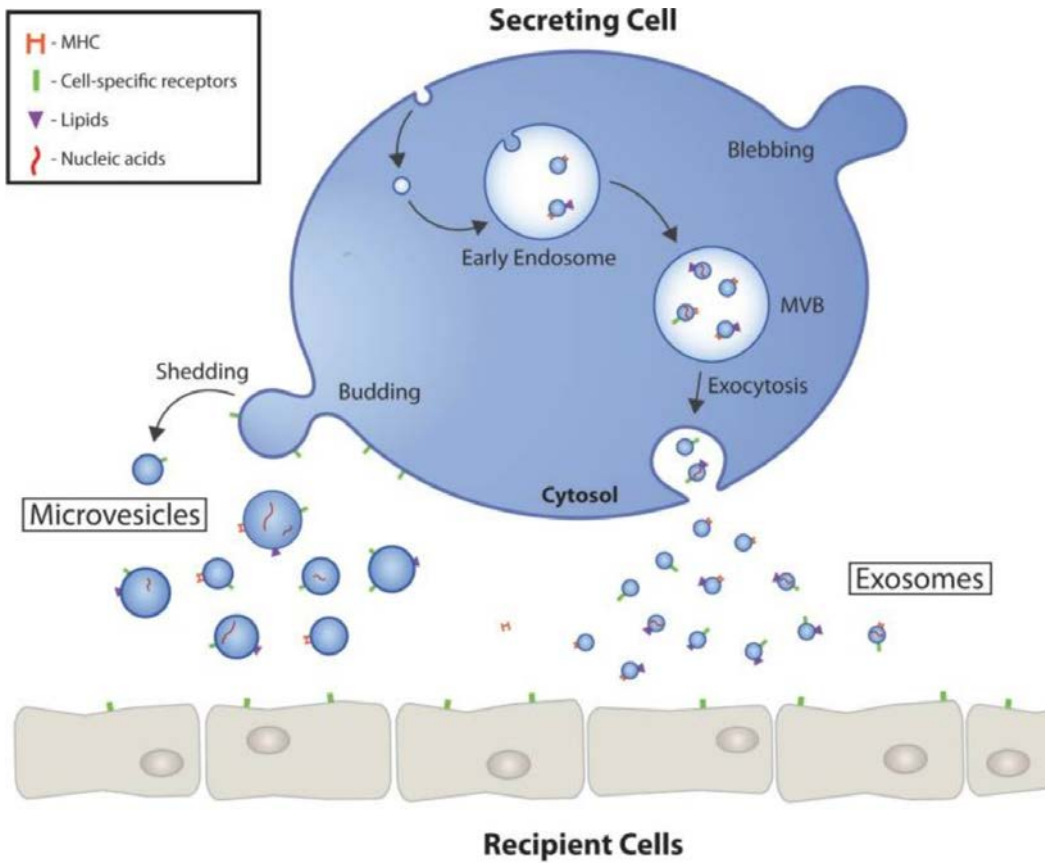

Figure 5. Schematic representation of extracellular vesicle production and secretion. *Extracellular vesicles can be divided into two main categories: microvesicles and exosomes. Both are membrane derived vesicles that can carry nucleic acids, and on their surface have cell-specific receptors and major histocompatibility complexes (MHC) (adapted from Gustafson et al., 2017).*

1.2.2. Neuroinflammation

Neuroinflammation is an inflammation of the nervous tissue, occurring as a complex biological response to stimuli, such as injury, autoimmunity, infection, or toxic molecules. Even though inflammation is primarily initiated as a beneficial response involved in tissue repair, if prolonged and uncontrolled, it could finally aggravate the disease through production of inflammatory mediators that inflict tissue damage.

It has been shown that almost all brain cells can secrete inflammatory mediators (Ramesh et al., 2013). Nevertheless, most immune functions in the brain are conveyed by microglia, innate immune cells that are constitutively present in the brain, sensing tissue damage and responding to it (Aschner, 1998). These cells are of myeloid origin and proliferate independently in CNS. Microglia are distributed throughout the brain, with the role of recognizing and reacting to subtle changes in the surrounding microenvironment. In physiological conditions, microglia serve in collecting debris and pathogens. However, in pathological conditions, microglia get stimulated through proinflammatory mediators, coming from brain, immune or vasculature cells. Hence, microglia alter from resting, ramified shaped cells to active microglia with amoeboid-like morphology (Hanisch, 2002). Accordingly, four major phenotypes of microglia in rodents can be distinguished: ramified, primed, reactive and amoeboid (Torres-Platas et al., 2014). After activation, microglia start to proliferate, upregulate MHC which is important for prolonged immune response, and secrete excessive amounts of proinflammatory cytokines, MMPs, as well as cytotoxic molecules, such as ROS and nitric oxide, NO (Colton and Gilbert, 1987; Konnecke and Bechmann, 2013; Liu et al., 2002; Sawada et al., 1989). Finally, microglia surround the inflammatory stimuli site and become phagocytic (Kreutzberg, 1996). Interestingly, microglia do not migrate to lymph nodes to present antigens, hence CNS parenchymal antigens get through interstitial fluid to draining lymph nodes (Engelhardt et al., 2016).

On the other hand, secreted microglia-derived inflammatory mediators stimulate astrocytes and immune cells to further produce inflammatory mediators. Further on they lead to chemotaxis of B and T lymphocytes, and their trafficking through the CNS barriers, sustaining inflammatory processes and leading to neuronal damage (Ransohoff and Brown, 2012). Neurons in turn secrete adhesion molecules and trophic factors, again attracting microglial cells and astrocytes that can release more trophic factors hence helping neurons in tissue repair (Ramesh et al., 2013). In conditions where inflammation is extended, continuous activation of glial, immune and vasculature cells can generate feedback loops that sustain inflammation thereby causing neural injury, and eventually neurodegeneration (Gendelman, 2002).

The complex picture of the involvement of different cytokines in these processes is hard to understand due to their pleiotropic effects, in both neuroprotection and neurodegeneration, *e.g.* IL-6 promotes neuronal survival in the process of reactive astrogliosis occuring in the brain injury, while it has been shown that increased levels of IL-6 negatively correlate with certain brain disorders, such as multiple sclerosis, Huntigton's disease or Alzheimer's disease (Erta et al., 2012). Similarly, TNF is found to be involved in neurotoxicity, and oligodendrocytes death, and inactivation of TNF has been shown to be beneficial in several diseases (Abo-Ouf et al., 2013; Kumar et al., 2010), while knocking out TNFR1 leads to exacerbation of the disease in a model of experimental autoimmune neuritis, suggesting anti-inflammatory role of TNF (Lu et al., 2007).

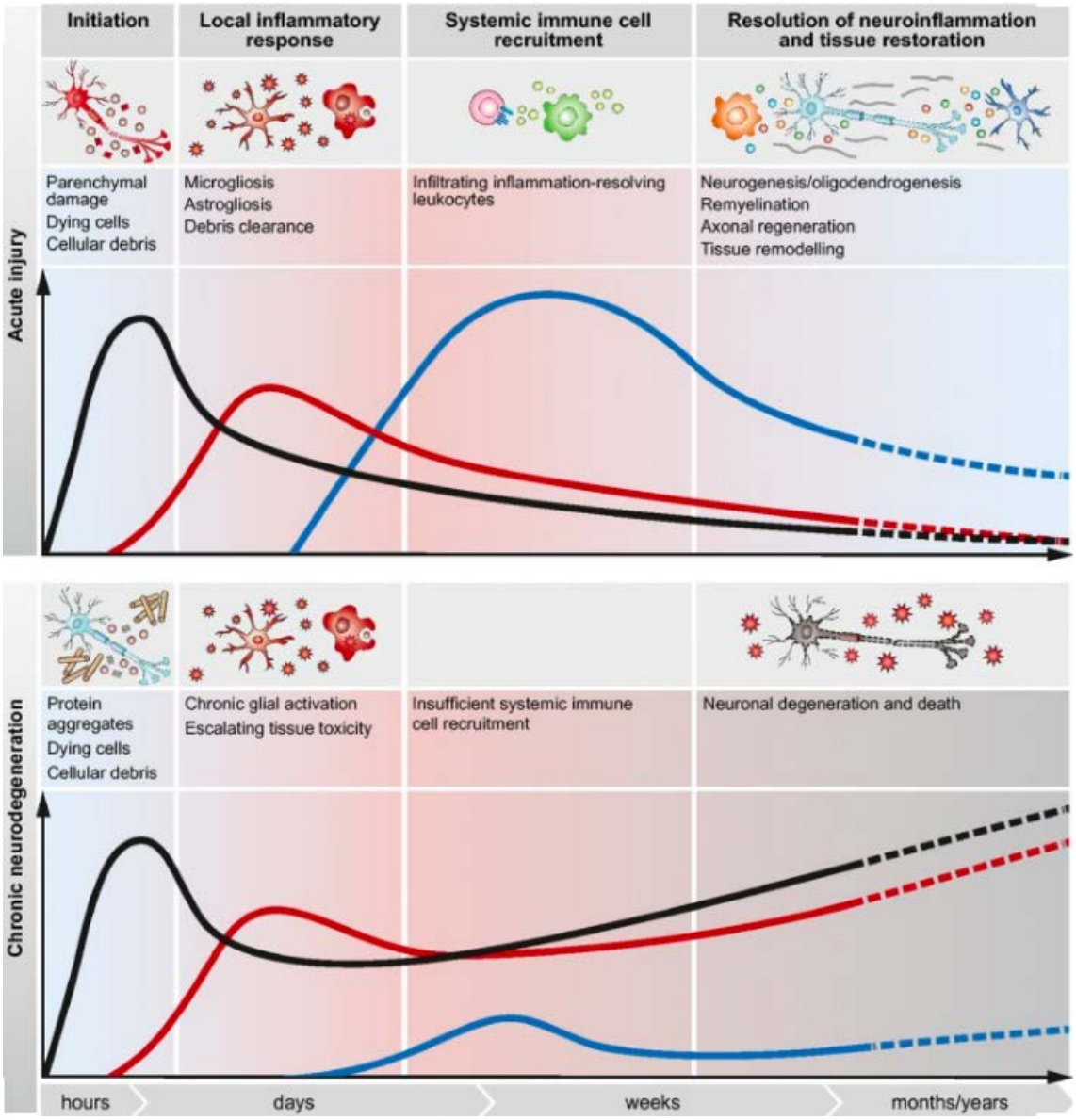

Figure 6. Inflammatory response to acute and chronic damage of central nervous system. *When damage to the CNS parenchyma (black line) occurs, glial cells get activated resulting in local inflammatory response (red line). In the next step, leukocytes are attracted to the site of damage (blue line), where they are involved in the resolution of the inflammation. If the resolution is not successful, it turns into chronic neuroinflammation, characterized by neurodegeneration (adapted from Schwartz and Baruch, 2014).*

1.2.2.1. Conditions associated with neuroinflammation

Neuroinflammatory disorders are conditions in which the response of the immune system causes damage to the CNS. It is observed in acute conditions, such as neuroinflammation associated to systemic inflammation, viral infections (such as HIV), traumatic brain injury (TBI) and stroke, as well as with aging and in various neurodegenerative diseases such as Alzheimer's disease (AD) and MS.

In neuroinflammatory conditions, highly complex inflammatory processes occur that are observed to share same properties among different disorders (Ghersi-Egea et al., 2018). The series of events include activation of microglia and CNS barrier constituents, leading to barrier opening and trafficking of immune cells, resulting in production of additional inflammatory mediators. These processes initiate secondary brain damage and chronic inflammation that lead to neurodegeneration, functional modulation of neurons, and finally behavioral changes (**Figure 6.**). Interestingly, prolonged inflammation occurs as a result of either persistence of the trigger of inflammatory response or due to unsuccessful resolution of inflammation.

1.2.2.2. Sepsis-associated neuroinflammation

Inflammation is considered systemic when the mechanisms that should occur locally around the source of inflammation (trauma, infection, burns, bleeding) shift to the systemic level. Systemic inflammation is usually linked with the systemic inflammatory response syndrome (SIRS) which is the complex pathophysiological body response to an infectious or noninfectious insult which appears as a result of uncontrolled immune response of the host, leading to release of high levels of proinflammatory cytokines and activation of immune cells and the neuroendocrine system (Tsiotou et al., 2005). SIRS can escalate into multiple organ dysfunction (MODS). SIRS induced by infection is called sepsis. Sepsis is defined as

life-threatening condition occurring as a result of dysregulated immune response to a pathogen that leads to injury and in severe cases to dysfunction of organs (Kesselmeier and Scherag, 2018). Septic shock is described as severe sepsis including acute circulatory failure with persistent arterial hypotension (Kang et al., 2015).

Sepsis affects millions of patients worldwide and presents the leading cause of death among hospitalized patients, predominantly in children (Liu et al., 2014). Sepsis survivors have shorter life expectancy and are left with poorer quality of life, due to frequent organ amputations. So far, no effective treatment has been found to treat SIRS.

In sepsis, immune system cells such as macrophages and DCs overproduce cytokines, which leads to stimulation of other immune cells, neutrophils and lymphocytes, aggravating the inflammatory response. It has been shown that TNF, IL-6, IFN-γ, IL-1β play an important role in these processes and that their neutralization can mitigate the severity of inflammatory response (Beutler et al., 2008; Hack et al., 1989; Heinzel, 1990; Ohlsson et al., 1990).

Interestingly, the brain is the first organ to be affected in sepsis and sepsis associated encephalopathy (SAE) is the most common complication observed in patients, often resulting in cognitive impairment (Chaudhry and Duggal, 2014; Sonneville et al., 2013). Although SAE is reversible, it has been shown that lifelong changes in behavior are observed in around half of the patients (Hopkins and Jackson, 2006). SAE is characterised by the decrease in cerebral blood flow, loss of brain barriers integrity, neuronal cell loss, alteration in neurotransmitters' expression and impairment in glial cell function (Papadopoulos et al., 2000). Although systemic inflammation starts in the periphery, the inflammation at one point spreads to the brain. In sepsis, transcription of pro- and anti-inflammatory cytokines in the brain is induced, such as TNF, IL1-β, TGF-β, MCP-1, leading to neuroinflammation (Semmler et al., 2008). These inflammatory mediators have been shown to be able to alter the expression of glutamate receptors on neurons, leading to brain dysfunction (Stellwagen and Malenka, 2006; Terrando et al.,

2010). Thus, sickness behavior, general feeling of being unwell, is present in SAE and it is related to the effects of proinflammatory cytokines (IL-1α, IL-1β, TNF and IL-6) on the brain centers involved in behavioral, neuroendocrine and autonomic responses (Heming et al., 2017). In mice models of systemic inflammation, it has been shown that microglia gets promptly activated and continuously secretes proinflammatory mediators (Henry et al., 2009; Semmler et al., 2005).

1.2.2.3. Neuroinflammation in Alzheimer's disease

Alzheimer's disease (AD) is the most common neurodegenerative disorder today. AD is the most prevalent in the age of 65 and older and around two-thirds of diseased are women. Neuronal damage occuring as a consequence of Alzheimer's disease is causing problems with different bodily functions, such as walking, talking, swallowing, etc., but it also leads to dementia. Dementia is characterized by decline in memory and in other cognitive abilities, ultimately disabling person to carry out normal daily activities. Although disease is diagnosed due to the memory problems (especially episodic memory), it is accepted that pathology of Alzheimer's disease begins decades before. The diagnosis is confirmed with the progression of decline in memory and other cognitive functions, which finally interfere with everyday life of the patient. The exact cause of AD remains elusive, but it is believed that the disease results from multiple factors, genetic, environmental and social (Masters et al., 2015). On a molecular level, AD is characterised by the neuronal cell loss and the decrease in dendritic arborization in cerebral cortex and subcortical areas. Moreover, presence of amyloid plaques, deriving from the accumulation of amyloid beta (Aβ), and neurofibrillary tangles (NFTs), resulting from the misfolding of Tau protein, can be found as well (Serrano-Pozo et al., 2011).

Several hypotheses about the cause of AD exist. However, this work is mostly based on the amyloid cascade hypothesis. It states that the main cause of AD is the Aβ peptide, derived from the defective processing of amyloid precursor

protein (APP) appearing in the onset of AD (Hardy and Allsop, 1991). Interestingly, mutations in the proteins presenilin 1 (PS1) and presenilin 2 (PS2), comprising catalytic active site of γ–secretase, can cause familial AD (Sherrington et al., 1995). Amyloid plaques as extracellular deposits of Aβ, are found in 90% of AD cases, although intriguingly, literature showed that they do not correlate significantly with the neuronal cell loss and dementia (DaRocha-Souto et al., 2011). Interestingly, more recently, the amount of soluble Aβ oligomers (AβO) has been shown to correlate more accurately with the disease progression (Viola and Klein, 2015). Additionally, soluble oligomeric Aβ, is observed to be able to induce neuronal cell death and impairment in synaptic transmission, causing behavioral changes (Selkoe, 2008). Also, in most transgenic mouse models cognitive impairment was observed before the onset of amyloid plaques, also suggesting the importance of conveying the research on precursor Aβ species (Dineley et al., 2002; Hartman et al., 2005; Webster et al., 2014).

Oligomeric Aβ derives from APP, a highly conserved protein, which is present in most tissues in our body. However, in the brain it is specifically found in neuronal synapses and its role remains elusive. APP is a single-pass transmembrane protein (type I) with a small cytoplasmic region and a large extracellular domain. APP can be cleaved in two ways, resulting in non-amyloidogenic or amyloidogenic pathway. Aβ fragment can vary in size, but mostly it is 40 amino acids (AA) long (Aβ1-40). However, in about 10% of the cases a 42 AA variant is produced called Aβ1-42 or Aβ42, which is hydrophobic and more commonly found in plaques (Seeman and Seeman, 2011).

The process of Aβ aggregation starts from Aβ peptides that aggregate into soluble oligomers, which group into regular β-sheets, called fibrils. Then the fibrils cluster together to form insoluble Aβ plaques (***Figure 7***).

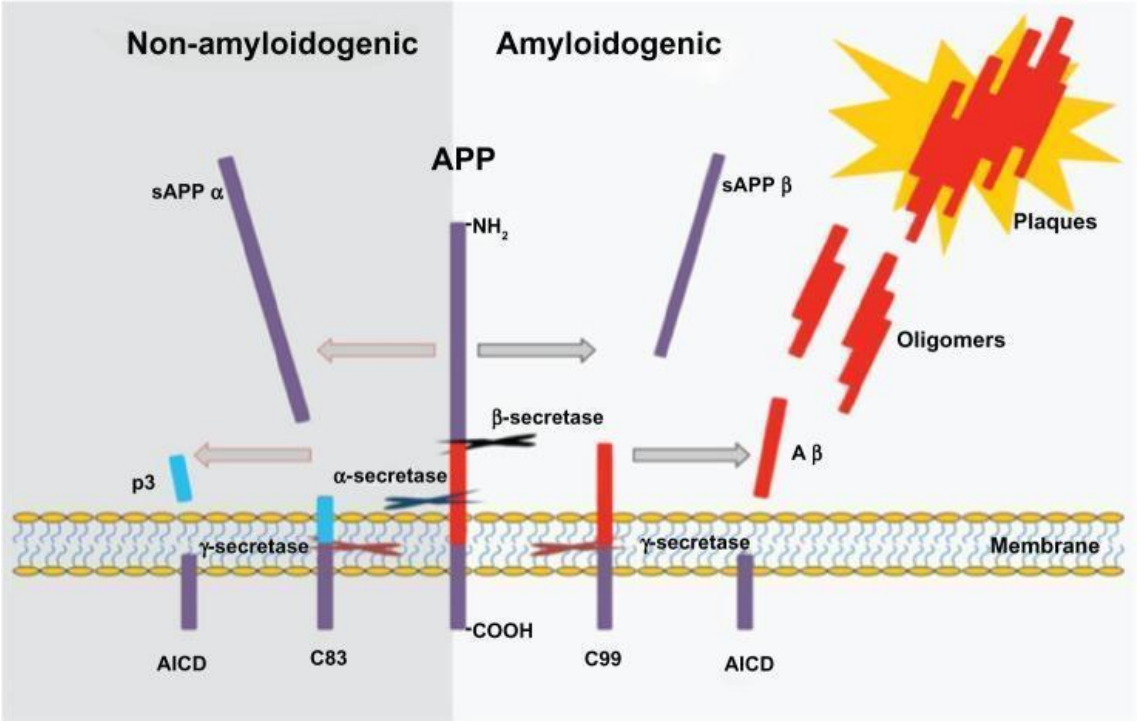

Figure 7. Schematic representation of proteolytic processing of APP. *Two pathways of proteolytic processing of APP exist. In the "amyloidogenic pathway" APP is first cleaved by β-secretase, and then by γ–secretase, generating Aβ. In the "non-amyloidogenic pathway, APP is first cleaved by α-secretase, and then by γ–secretase, generating p3 (adapted from Chen et al., 2013).*

Nowadays, it has been established that next to amyloid species and NFTs, also neuroinflammation plays an important role in progression of AD.

Activated microglia are clustered around amyloid plaques in the brain of AD patients, even before the development of AD clinical picture (Cagnin et al., 2001; Meda et al., 1995; Sasaki et al., 1997). It has been observed that when active, microglia overproduce oxygen radicals and inflammatory mediators which are shown to be highly neurotoxic (McGeer and McGeer, 2000). Also, Aβ has been observed to be able to activate microglia directly, and thus exert indirect proinflammatory effects, inducing secretion of proinflammatory mediators, such as NO, TNF, and superoxides (Combs et al., 2000; Qin et al., 2002). Activated microglia and astrocytes can lead to the release of Tau protein aggregates from dying neurons, which contribute further to the activation of glial cells and neuronal cell

death, perpetuating neuroinflammation. On the contrary, microglia were shown to be protective in the early stages of AD, conveying the clearance and phagocytosis of Aβ. *In vitro* studies confirmed that microglial cells can remove Aβ peptide (Hardy and Selkoe, 2002). Also, microglia secrete proteolytic enzymes which are able to degrade Aβ (Heneka et al., 2015). In a physiological immune system reaction, after being activated, microglia are involved in clearing of pathogens by phagocytosis and by production of cytokines. However, in AD, since Aβ is produced continuously, it leads to perpetuation of inflammation (Walters et al., 2016).

1.2.2.4. Role of CP in neuroinflammation

In physiological conditions, the only residing immune cells in the brain parenchyma are microglial cells, while T or B cells can be found solely in CSF, i.e. in ventricles and in subarachnoid spaces. Since various immune cells have been observed on both sides of the BCSFB, the role of CP in immunity has been speculated (Schwerk et al., 2015; Strominger et al., 2018). At the CP, Kolmer's epiplexus cells are positioned on the apical side of the CPE cells, targeting antigens from CSF. On the other side, in CP stroma, DCs can be found, which are able to protrude to ventricles, and scavenge antigens (Ghersi-Egea et al., 2018; Nathanson and Chun, 1989). In healthy conditions, in CP stroma, mostly CD4+ T cells, specific for brain self-antigens, can be found, and their role has been implicated in neurogenesis, neuroprotection and spatial learning (Kaur et al., 2016). CPE cells constitutively express MHC class I and adhesion molecules (*e.g.* vascular cell adhesion protein 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1)), as well as chemokines (*e.g.* MCP-1 and MIP-1β), to facilitate leukocyte trafficking. Expression of these molecules was shown to be upregulated during inflammatory processes (Wolburg et al., 1999). On the other hand, DCs from CP stroma, through secretion of IL-10 may silence present T cells, thus limiting their response. Also, in various conditions associated with neuroinflammation, such as viral infections (eg. HIV), TBI, stroke, systemic inflammation, MS, and AD, T cells, monocytes and

neutrophils have been observed to paracellularly migrate between CPE cells (Meeker et al., 2012; Szmydynger-Chodobska et al., 2012). It has been shown that a large number of T cells that enter this way are inflammation resolving cells that contribute to the resolution of the neuroinflammation (Schwartz and Baruch, 2014).

Since, CPE cells are connected with TJs that prevent leakage of undesired molecules and pathogens to the brain, they need to be either cleaved or relocated in order to permit leukocytes entry to the brain. This results in the increase in barrier permeability and morphological alternation of CPE cells observed in *e.g.* stroke, TBI, and MS (Brkic et al., 2015a; Engelhardt et al., 2001; Johanson et al., 2011; Renu et al., 2017). The process of cleavage of TJs is attributed to MMPs amongst others, and their upregulation is observed in both, CSF and blood, in above mentioned diseases (Grossetete et al., 2009; Ramos-Fernandez et al., 2011; Yong et al., 2007). Moreover, CPE cells were shown to be able to secrete MMPs, and their secretion is promoted by cytokines (Mun-Bryce and Rosenberg, 1998). Besides the role in leukocyte trafficking into the brain, the CP exhibits other characteristics of a tissue involved in brain immunity. Capillaries bellow CPE cells are fenestrated, which allows five times higher blood flow than in parenchyma. This feature enables CP to "sense" inflammation and transmit this inflammatory message from periphery to the CNS, as well as within CNS (Maktabi et al., 1990; Schwerk et al., 2015). CPE cells, through the extended surface with numerous microvilli and expressed receptors for cytokines, hormones, growth factors, neurotransmitters and toxins, located on both apical and basal parts of cells, is equipped to integrate signals deriving from blood, nervous and immune systems, and swiftly adjust its transcriptome, proteome and secretome to be able to respond to changes in the microenvironment. It has been shown that these changes are mostly in upregulation of extracellular matrix remodelling genes implicated in facilitating the access of immune cells to the brain and the downregulation of TJ genes (Marques et al., 2009a; Szmydynger-Chodobska et al., 2009). Interestingly, all mentioned alterations in transcriptome are shown to be transient, returning to normal state in several days. Moreover, it has been shown

that the CP acts similarly in terms of gene expression after both, acute and chronic inflammatory stimuli, though in the latter the effect is milder (Marques and Sousa, 2015).

Since the CP is a main production site of CSF, various proteins, mRNAs and miRNAs that are produced by the CP are also secreted directly into this fluid, which facilitates distribution of these biologically active molecules throughout the whole CNS. Through this route CP-derived cytokines are able to exert their paracrine effects on brain cells, as well as on other CPE cells, and to initiate a cascade of events, either enhancing or mitigating neuroinflammation. Hence, testing CSF composition could reveal the occurrence of a specific disease, and the development of tests for CSF biomarkers present a promising diagnostic potential (Demeestere et al., 2015). Also CP-derived EVs are considered as a potential therapeutic platform for several brain pathologies (Ramirez et al., 2018). Interestingly, transplantation of a healthy CP was previously considered as a possible therapeutic approach, and was showen to be successful in treating both acute and chronic brain diseases (Ide et al., 2001; Skinner et al., 2006).

Until today, the exact functional and structural changes occurring in CP in the initiation of neuroinflammation are not fully elucidated. Taking into account that even small alterations in function or structure of the CP can have notable consequences on the brain functioning, understanding the contribution of these processes to neuroinflammation might lead to the development of new therapeutic targets.

2. Goals

Considering the rising evidence of the role of the choroid plexus (CP) in neuroinflammatory processes through alterations of morphology and functionality of the choroid plexus epithelial (CPE) cells, the main goal of this thesis is to study the contribution of the CP in the initiation of neuroinflammation in two different animal models of diseases associated with neuroinflammation. The first model is a mouse model of systemic inflammation, namely, lipopolysaccharide (LPS)-induced sepsis in which the inflammatory signal derives from periphery. The second model is a more recent mouse model of Alzheimer's disease, namely intracerebroventricular (i.c.v) injection of Aβ oligomers (AβO) in which the inflammatory signal originates from the central nervous system (CNS). According to this, we have defined sets of goals with specific tasks:

(1) Examine blood-cerebrospinal fluid (BCSFB) functionality and underlying morphological changes of CPE cells in the initiation of neuroinflammation in the model of i.c.v. AβO injection. Changes in BCSFB permeability, the morphology of CPE cells and gene and protein expression of tight, adherens and gap junctions as the structural constituents of the BCSFB upon i.c.v. injection of AβO will be investigated.

(2) Define whether changes in BCSFB permeability precede changes in BBB permeability in the initiation of neuroinflammation, through assessment of BBB permeability upon i.c.v. AβO injection.

(3) Assess the effect of i.c.v. AβO injection on behavior of the animals by short and long term memory testing**.**

(4) Assess the mechanisms that lead to changes in BCSFB permeability upon i.c.v. AβO injection by analysing gene expression of several matrix metalloproteinases (MMPs) in the CP, analysing MMP activity in the CSF and analysing MMP-3 contribution to AβO-induced BCSFB permeability using broad spectrum MMP inhibitor and MMP-3 deficient mice.

(5) Examine functional changes of the CP during the initiation of neuroinflammation after i.c.v. AβO injection and intraperitoneal (i.p.) LPS injection, that are reflected in secretory activity of CPE cells. In order to accomplish this, gene and protein expression of transthyretin (TTR) in the CP, brain derived neurotrophic factor (BDNF) gene expression in the CP and its concentration in CSF, cytokine expression in the CP, hippocampus and cerebrospinal fluid (CSF), the amount of extracellular vesicles (EVs) in CSF, and study gene expression of the EV marker CD63 and miR-155 in the CP will be analyzed upon i.c.v. injection of AβO and/or i.p. injection of LPS.

(6) Define whether microglial activation precedes secretion of proinflammatory cytokines from CPE cells in the initiation of neuroinflammation. To address this, the number of activated microglial cells around ventricles upon i.c.v. AβO injection will be studied.

3. Materials and methods

3.1. Animals

Female C57BL/6 mice, age from 8-10 weeks were purchased from Janvier (Le Genest-Saint-Isle, France), while MMP3-deficient mice (C57BL/6 background) were bred in VIB-UGent Center for Inflammation Research animal facility. All animals were housed in specific pathogen-free (SPF) facility in groups of 4-6 per cage, with *ad libitum* access to water and food and a 14 h light and 10 h dark cycle. All experiments were approved by the Ethical Committee for the Use of Laboratory Animals of the Faculty of Sciences of Ghent University (EC 2013-077).

3.1.1. Animal model of systemic inflammation

Endotoxemia was used as mouse model of systemic inflammation (Fink, 2014). Briefly, endotoxemia was induced by intraperitoneal (i.p.) injection in mice with lipopolysaccharide (LPS) derived from *Salmonella enterica serotype abortus equi* (Sigma) dissolved in phosphate-buffered saline (PBS). The dose administered was 200 µg/ 20 g body weight (the LD_{100} dose for C57BL/6 mice). Control mice were i.p. injected with PBS. Animals were sacrificed at 2 and 6 h time points.

3.1.2. Animal model of Alzheimer's disease (AD)

The AβO injection model was used as a mouse model of AD. Animals were divided into two experimental groups: mice injected with Aβ1-42 oligomers (AβO) and mice injected with scrambled Aβ 1-42 oligomers.

AβO preparation was based on Kuperstein et al. (2010). In short, Aβ1-42 (rPeptide; #A-1163-1) or scrambled Aβ1-42 (rPeptide; #A-1004-1) was dissolved

in hexafluoroisopropanol (HFIP; Sigma-Aldrich; #105228) at a final concentration of 1 mg/ml. HFIP was then removed by a SpeedVac vacuum concentrator. The peptide film left in the tube was further dissolved in DMSO (Sigma-Aldrich; #D4540) at a final concentration of 1 mg/ml. Monomeric peptide was next purified using a 5 ml HiTrap desalting column (GE Healthcare; #17-408-01) followed by elution with Tris-EDTA buffer (50 mM Tris and 1 mM EDTA, pH 7.5). Peptide concentration was determined by Thermo Scientific–Pierce Micro BCA Protein Assay (#23225), according to the manufacturer's instructions. The peptide was then incubated at room temperature (RT) for 2 h and diluted in Tris-EDTA buffer to a final concentration of 1 μg/ml.

The prepared AβO or scrambled peptide was injected intracerebroventricularly (i.c.v.). Prior to performing the injection procedure, mice were anesthetized with isoflurane and placed in a stereotactic frame. Heating pad was used to maintain mouse body temperature at 37°C. Injection coordinates were determined using the Franklin and Paxinos mouse brain atlas. The hole in the skull was made with surgical drill anteroposterior 0.07, mediolateral 0.1, when measured from Bregma, and dorsoventral coordinate was 0.3. Using a Hamilton needle, a volume of 5 μl (1 μg/ml peptide) was injected in the right lateral cerebral ventricle.

In specific experiments, the i.c.v. injection of AβO or scrambled peptide was combined with i.c.v. injection of 1 μg of the broad spectrum MMP inhibitor GM6001 (Merck; #CC1100) dissolved in DMSO.

3.1.3. Behavioral testing

Animal behavior was tested using novel object recognition (NOR) test described by Antunes and Biala (Antunes and Biala, 2012). The NOR test is based on the innate tendency of rodents to preferentially explore novel objects over familiar ones. Mice were tested in a rectangular, clear open-field area made from

acrylic (40 × 40 × 40 cm). The protocol consisted of three phases: habituation, training, and testing. On the first day, before AβO i.c.v. injection, animals were placed for 5 min into the empty open-field arena to habituate to the environment. On the second day, the training phase was performed. Mice were positioned in the arena facing the wall opposite to two identical objects which were placed at two opposite sides in the box at the same distance from the nearest corner. Mice were allowed to freely explore the objects (A1 and A2) for 5 min and then were returned to their home cage. In the testing phase, short term memory (STM) was tested 15 min after training phase. After replacing one of the familiar objects (A1 or A2) by a novel one (A3 or A4), mice were put back in open-field arena and left for 5 min to freely explore. On the third day, 24 h after the training session, long term memory (LTM) was assessed. After replacing one of the familiar objects (A1 or A2) by a novel one (A5 or A6), mice were put back in open-field arena and left for 5 min to freely explore. To exclude the existence of olfactory clues, all objects were thoroughly cleaned with 20% ethanol after each trial. The exploration of the objects was considered as deliberate contact with their mouth or nose with any object. The exploration time for both objects during the test phase was recorded. The preference for the novel object was calculated using the discrimination index (D): D = [novel object exploration time-familiar object exploration time/(novel object exploration time + familiar object exploration time)].

3.2. CSF isolation

Two and 6 h after i.c.v. injection, mice were sedated with 200 µl of ketamine/xylazine (20 mg/ml/0.002%). CSF was isolated using cisterna magna puncture method described by Liu and Duff (2008). Special needles were made from borosilicate glass capillary tubes (B100-75-15, Sutter Instruments) on the Sutter p-87 flaming micropipette puller (pressure 330 Pa, heat index 300). Cisterna magna was exposed and CSF was collected by inserting needle into fourth ventricle.

3.3. BCSFB and BBB permeability

The BCSFB and BBB permeability was assessed according to the method described by Vandenbroucke et al. (2012). One hour before CSF was collected, 4 kDa FITC-dextran (Sigma-Aldrich; #46944) was injected intravenously (i.v.) at a concentration of 75 mg/kg. After CSF isolation, mice were transcardially perfused using D-PBS/heparin (0.2% heparin) to remove all dextran from circulation. Brain tissue was isolated. CSF samples were diluted 100-fold in sterile D-PBS. BCSFB leakage was assessed by measurement of fluorescence at $\lambda_{ex}/_{em}$ 488/520 nm on fluorometer. For BBB leakage determination, brain samples were cut into small pieces and incubated at 37°C overnight in formamide on shaker. The next day, samples were centrifuged for 15 min at 12000 rpm and supernatant was collected and diluted twofold in sterile D-PBS for further analysis. BBB leakage was assessed by measurement of fluorescence at $\lambda_{ex}/_{em}$ 488/520 nm on fluorometer.

3.4. Cytokines and BDNF measurement in CSF

Cytokines (IL-1α, IL-1β, IL-6, IL-10, IL-12p40, IL-13, IL-17, TNF-α, IFN-γ, Rantes, MCP-1, MIP-1a, MIP-1b, GMCSF) levels in CSF were measured using the Bio-Plex cytokine assays - 23plex (Bio-Rad; #M60009RDPD) according to the manufacturer's instructions. In short, CSF samples were diluted 15 times in 0,5% bovine serum albumin (BSA)/sample diluent and left to incubate for 20 min. Afterwards, the plate was wetted with Essay buffer. Standard was reconstituted and diluted in a dilution series. Next, antibody coupled capture beads were prepared, vortexed and plated. Diluted samples and standards were added in duplicates in wells and samples were incubated for 30 minutes. After washing, detection antibodies were added to each well, and incubated again for 30 minutes. Again, after washing, streptavidin-PE solution is added to the wells, and incubated for 10 minutes. Finally, beads were resuspended in assay buffer. For reading,

MagPix (Luminex Corporation) was used. The absolute concentration of the samples were calculated using standard curve for each sample.

For BDNF concentration measurement, CSF was diluted 15 times in dilution buffer +0.01% BSA (carrier protein), and BDNF concentration was measured using the Bio-Plex growth factor assays (Merck Chemicals N.V., BDNF Mouse Magnetic Endocrine Panel - 1 Plex, MPTMAG-49K, Milliplex) according to the manufacturer's instructions.

3.5. Analysis of matrix metalloproteinase (MMP) activity in CSF

MMP activity in the CSF was assessed with the Omni-MMP fluorogenic substrate kit according to manufacturer's instructions (Enzo Life Sciences; #BML-AK016). Briefly, CSF samples were diluted 50-fold in MMP peptide fluorogenic assay buffer. MMP activity was determined by measuring the increase in fluorescence at $\lambda_{ex}/_{em}$ 320/460 nm on fluorometer (Omega Fluorostar).

3.6. Extracellular vesicle (EV) analysis

To quantify the amount of EVs in CSF, cell debris was first removed by centrifugation at 300 g. Equal volumes of CSF were taken and diluted 1/100 in PBS. Samples were injected into the NanoSight LM10-HS instrument (NanoSightLtd) and three 60 sec videos were recorded for each sample. Analysis was done using Nanoparticle tracking analysis (NTA) software version 2.3 to determine concentration and size of measured particles with corresponding standard error. NTA post-acquisition settings were optimized and kept constant between samples. Absolute numbers were recorded and back-calculated using the dilution factor.

3.7. Gene and miRNA expression analysis

3.7.1. Tissue isolation

Preparation of the tissue for RNA and protein analysis involved transcardial perfusion of mice with D-PBS/heparin (0,2% heparin) supplemented with 0,5% bromophenol blue to help visualize the choroid plexus tissue. Brain was dissected from the skull and choroid plexus was obtained from all four ventricles and snap frozen in liquid nitrogen. Next, the hippocampus was taken and immersed in RNAlater (Ambion; #AM7020) for further analysis.

3.7.2. RNA isolation

Total RNA was isolated with the (mi)RNeasy kit (Qiagen; #74106 and #217004) according to manufacturer's instructions. Total RNA was isolated without pooling of samples. RNA concentration and purity were determined spectrophotometrically using the Nanodrop Technologies ND-1000.

3.7.3. Gene expression analysis

For gene expression analysis, cDNA was synthesized using the iScriptTM cDNA Synthesis Kit (Bio-Rad; #172-5038) with 500 -1,000 ng starting material according to manufacturer's instructions. Real-time qPCR was done on the Light Cycler 480 system (Roche) using the LightCycler 480 SYBR Green I Master mix (Roche; #04887352001) or the SensiFAST SYBR No-ROX Kit (Bioline; #BIO-98002) according to manufacturer's instructions. Two to three most stable

housekeeping genes were determined for each experiment with geNorm software (Vandesompele et al., 2002) and were used to normalize gene expression levels. All primer sequences can be found in ***Table 2***. The reference genes for choroid plexus samples were *Hprt*, *Ubc* and *Rpl*.

Table 2. *Forward and reverse primers sequences used for gene expression analysis.*

Gene	Forward primer	Reverse primer
Cldn-1	TCTACGAGGGACTGTGGATG	TCAGATTCAGCAAGGAGTCG
Cldn-5	GCAAGGTGTATGAATCTGTGCT	GTCAAGGTAACAAAGAGTGCCA
Ocln	CCAGGCAGCGTGTTCCT	TTCTAAATAACAGTCACCTGAGGGC
Zo1	AGGACACCAAAGCATGTGAG	GGCATTCCTGCTGGTTACA
Cx43	ACAGCGGTTGAGTCAGCTTG	GAGAGATGGGGAAGGACTTGT
N-cdh	TCCTCTGCATCCTCACTATCACA	GTAAGTGACCAACTGCTCGTGAAT
E-cdh	TCGGAAGACTCCCGATTCAAA	CGGACGAGGAAACTGGTCTC
Ttr	TGGACACCAAATCGTACTGGAA	CATCCGCGAATTCATGGAA
Bdnf	TTACCTGGATGCCGCAAACAT	TGACCCACTCGCTAATACTGTC
Il1β	CACCTCACAAGCAGAGCACAAG	GCATTAGAAACAGTCCAGCCCATAC
Il6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
Tnf	ACCCTGGTATGAGCCCATATAC	ACACCCATTCCCTTCACAGAG
Mmp3	AGTCTACAAGTCCTCCACAG	TTGGTGATGTCTCAGGTTCC

Mmp8	ATTCCCAAGGAGTGTCCAAGC	TGATTGTCATATCTCCAGCACTGG
Mmp9	CTGGACAGCCAGACACTAAAG	CTCGCGGCAAGTCTTCAGAG
Cd63	GAAGCAGGCCATTACCCATGA	TGACTTCACCTGGTCTCTAAACA
Hprt	AGTGTTGGATACAGGCCAGAC	CGTGATTCAAATCCCTGAAGT
Rpl	CCTGCTGCTCTCAAGGTT	TGGTTGTCACTGCCTGGTACTT
Ubc	AGGTCAAACAGGAAGACAGACGTA	TCACACCCAAGAACAAGCACA

Abbreviations: Cldn-1 Claudin-1*, Cldn-5* Claudin-5, *Ocln* Occludin, *Zo1* Zonula Occludens 1, *Cx43* Connexin-43, *N-cdh* N-cadherin, *E-cdh* E-cadherin, *Ttr* Transthyretin, *Bdnf* Brain derived neurotrophic factor, *Il1β* Interleukin *1β, Il6* Interleukin 6, *Tnf* Tumor necrosis factor, *Mmp3* Matrix metalloproteinase 3, *Mmp8* Matrix metalloproteinase 8, *Mmp9* Matrix metalloproteinase 9, *Hprt* Hypoxanthine-guanine phosphoribosyltransferase, *Rpl* Ribosomal protein L, *Ubc* Ubiquitin C.

3.7.4. miRNA expression analysis

Firstly, cDNA was synthesized using MultiScribeTM Reverse Transcriptase (InvitrogenTM) kit, starting from equal amounts of 350 ng of total RNA (350 ng) isolated from choroid plexus. Real-time qPCR was done using TaqMan qPCR assays according to the manufacturer's instructions. miR-155 (ThermoFischer Scientific, 002571) expression was assessed. Two most stable miRNAs were determined for each experiment using the geNorm software (Vandesompele et al., 2002) and were used to normalize miRNA expression levels. miR-24 (ThermoFischer Scientific, 0024944) and miR-126 (ThermoFischer Scientific, 002228) were used as reference miRNAs. All primer sequences listed in ***Table 3***.

Table 3. *Forward and reverse primers sequences used for miRNA expression analysis.*

miRNA	Forward primer	Reverse primer
miR-24	CTAGCCTGCAGGCGTGCTGACCAC CCTTAAGT	ATCCGGCCGGCCGAAAGGCTCTA CAGACAAGG
miR-126	CTAGCCTGCAGGACAGCAGGTAAA CTTGCCTT	ATCCGGCCGGCCCCTGTTCTAGC ACATCAACC
miR-155	GACTGTTAATGCTAATCGTGATAG	GTGCAGGGTCCGAGGTATTC

3.8. Histological analysis

3.8.1. Tissue preparation

For cryosections, animals were perfused with PBS and brains were dissected out and immersed in cryoprotectant (Thermo Scientific; #4583) filled in cryomolds and frozen at -80°C until cryosectioned. For paraffin sections, animals were perfused with 4% PFA, and brains were fixed in 4% paraformaldehyde (PFA) overnight. The next day, brains were transferred into 79% ethanol for further storage. Paraffin embedding was performed in series of alcohol (70%, 80%, 95% and 100% and Xylene), followed by immersion in paraffin wax (56-60 ºC). Finally, brains were embedded into paraffin blocks.

Cryosections were cut 30 µm thick in a cryostat (Thermo ScientificTM; #HM500) and mounted on slides. Slides were let to air dry for 2 hours at RT, and then were fixed with 1% PFA for 10 min, followed by 3 x 5 min washing in PBS and 10 min of permeabilization with 0,1% NP-40. After two washes with PBS, samples

were blocked for one hour with 5% BSA (Sigma-Aldrich; catalog #A2153) on RT. Incubation with primary antibody was done overnight at 4°C and different dilutions were used for different antibodies, as listed in Table 4. The next day, slides were washed 3 times for 5 min in PBS and then for 90 min at room RT with secondary antibody diluted in 5% BSA (as listed in Table 4.). Sections were counterstained with Hoechst 33342 (1 µg/ml) and mounted with aqueous mounting media. A Leica TCS SP5 II confocal microscope was used for visualization.

Sections from paraffin-embedded tissue, were cut 4 µm thick on microtome (HM360, Prosan) in sagittal orientation, dewaxed and rinsed in water and PBS before staining. For antigen retrieval, citrate buffer (Dako; #S2031) was used and slides were washed 3 times for 5 min in PBS before overnight incubation with 0.01M NaBH4 (Sigma-Aldrich; #452882) to further reduce autofluorescence. The next day, slides were rinsed in PBS, and tissue was permeabilized with 0.05% Tween-20 (Sigma-Aldrich; #P1379) for 30 min at RT. Blocking of the samples was done with 2% BSA for 30 min at RT, followed by incubation for 90 min at RT with E-cadherin primary and corresponding secondary antibody diluted in 5% BSA (Table 4.). For IBA1 staining, incubation with 0.01M NaBH4 was skipped and slides were overnight incubated at 4°C with primary antibody (***Table 4***). On the second day, after washing, slides were incubated with LSAB2 System HRP and visualization was done using DAB chromogen. Final steps included dehydration and use of xylene-based mounting medium to preserve sections. Visualization of the staining was done using Olympus BX51 microscope. IBA1-positive cells were counted in predefined area of the brain, including both the cortex and hippocampus, using Fiji (http://fiji.sc/Fiji). Microglia were classified into resting and activated according to adopted criteria by Hains and Waxman (2006).

Table 4. Primary and secondary antibodies used with corresponding dilutions.

Primary Antibody	Data	Dilution	Secondary Antibody	Data	Dilution
OCLN	Life Technologies 33-1500	1:100	Goat anti-mouse	Dylight 594 Thermo Scientific	1:1000
ZO1	Invitrogen 617300	1:100	Goat anti-rabbit	Dylight 594, Thermo Scientific	1:1000
TTR	Dako A0002	1:200	Goat anti-rabbit	Dylight 594 Thermo Scientific	1:1000
ECDH	BD Transduction Laboratories 610181	1:500	Goat anti-mouse	Alexa Fluor 56 Life Technologies	1:500
IBA1	Wako 01919741	1:1000	LSAB2 System HRP	DAKO K0672	

Abbreviations: OCLN Occludin, ZO1 Zonula Occludens 1, TTR Transthyretin, *E-cdh* E-cadherin, IBA1 Ionized calcium-binding adapter molecule 1

3.9. Morphological analysis of choroid plexus tissue using serial block-face scanning electron microscopy (SBF-SEM)

For SBF-SEM, choroid plexus tissue was dissected and immediately transferred into fixative buffer (2% paraformaldehyde, Sigma-Aldrich; 2.5% glutaraldehyde, Electron Microscopy Sciences in 0.15 M cacodylate buffer, pH 7.4). The next day, samples were washed 3 times 5 min in cacodylate buffer, than osmicated in 2% osmium (EMS), 1.5% ferrocyanide, and 2 mM CaCl2 in cacodylate buffer for 1 h on ice. Samples were washed extensively in ultrapure water (UPW) and incubated in 1% thiocarbohydrazide for 20 min. After another turn of washes in UPW, a second osmication in 2% osmium in UPW was done for 30 min. The samples were washed again 5 times 3 min in UPW and placed in 2% uranyl acetate at 4°C overnight. The following day, samples were stained with Walton's lead aspartate stain for 30 min at 60°C. For this step, a 30 ml aspartic acid solution was used to freshly dissolve lead nitrate (final concentration 20 mM, pH 5.5). After incubation for 30 min at 60°C, the solution was filtered. After washing was performed 3 times for 5 min followed by dehydration using a series of ice-cold solutions of increasing ethanol concentration (30%, 50%, 70%, 90%, and twice 100%), followed by two dehydration steps of 30 min in 100% acetone. Subsequent infiltration with resin (Durcupan; EMS) was done by first incubating the samples in 50% resin in acetone for 4 h, followed by at least 5 changes of fresh 100% resin (including 2 overnight incubations). Next, samples were embedded in fresh resin and dried in the oven at 65°C for 72 h. The resin-embedded samples were mounted on an aluminum specimen pin (Gatan) using conductive epoxy (Circuit Works). The specimens were trimmed in a pyramid shape using an ultramicrotome (Ultracut; Leica) and the block surface was trimmed until smooth and at least a small part of tissue was present at the block face. Samples were coated with 5 nm Pt in a Quorum Q 150T ES sputter coater (Quorum Technologies). The aluminum pins were placed in the Gatan 3View2XP in a Zeiss Merlin SEM for imaging at 1.6 kV with a Gatan Digiscan II ESB detector. The Gatan 3view2XP was set to section 300 sections of 70 nm. IMOD (http://bio3d.colorado.edu/imod/) and Fiji

(Schindelin et al., 2012) were used for the registration of the 3D image stack and conversion to TIFF file format. Representation of the cell in 3D movies and snapshots were done in Imaris (BitPlane). 3D modeling of choroid plexus epithelial cells was performed using IMOD software.

3.10. Statistical analysis

Data are presented as mean ± standard error of mean (SEM). Data were analyzed by Student's t-test using GraphPad Prism program. Significance levels are indicated on the graphs: *$0.01 \leq p < 0.05$; **$0.001 \leq p < 0.01$; ***$0.0001 \leq p < 0.001$; ****$p < 0.0001$.

4. Results

4.1. The effect of intracerebroventricularly injected Aβ 1-42 oligomers (AβO) on blood-cerebrospinal fluid barrier (BCSFB) functionality and choroid plexus epithelial (CPE) cell morphology

4.1.1. The effect of i.c.v. injected AβO on BCSFB integrity

The impact of i.c.v. Aβ 1-42 oligomers (AβO) injection on blood-cerebrospinal fluid barrier (BCSFB) integrity after 2 and 6 h was evaluated using i.v. injection of 4 kDa FITC-dextran. In healthy conditions, this in case of an intact BCSFB, 4 kDa FITC-dextran is unable to cross the BCSFB and leak into the CSF. Consequently, the amount of 4 kDa FITC-dextran in the CSF is a measure for BCSFB integrity. The data presented in **Figure 1** show that AβO are able to induce increased permeability of the BCSFB 6 h after i.c.v. injection, observed as a significant increase in 4 kDa FITC-dextran levels in the CSF of injected mice in comparison to mice intracerebroventricularly (i.c.v.) injected with scrambled peptide as a control (95%, $p \leq 0.01$).

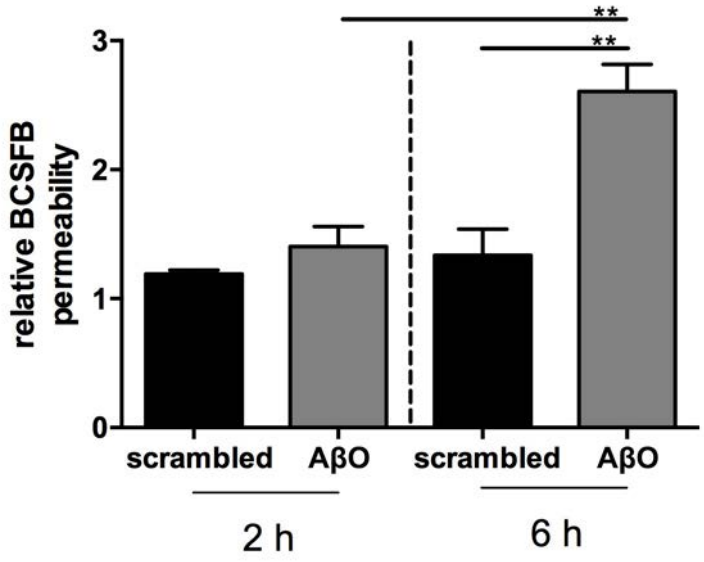

Figure 1. Relative BCSFB permeability after AβO i.c.v. injection. Relative ratio of i.v. injected 4 kDa FITC-dextran concentration in CSF 2 and 6 h after i.c.v. injection of AβO (grey) compared to scrambled control (black).

4.1.2. The restoration of BCSFB integrity after AβO injection in cerebral ventricles

In order to determine whether the observed change in BCSFB permeability is transient, the relative BCSFB permeability at 24 h, 48 h and 7 days after i.c.v. AβO injection, was tested, using the above mentioned method. The results represented in **Figure 2** show that the peak in BCSFB permeability was 24 h after AβO injection (5-fold increase, $p \leq 0.05$), while this was restored at later time points. Indeed, the BCSFB leakage at 48 h was not significantly higher then in case of scrambled injected mice. Moreover, when BCSFB permeability was measured 7 days after AβO injection, values returned to basal levels.

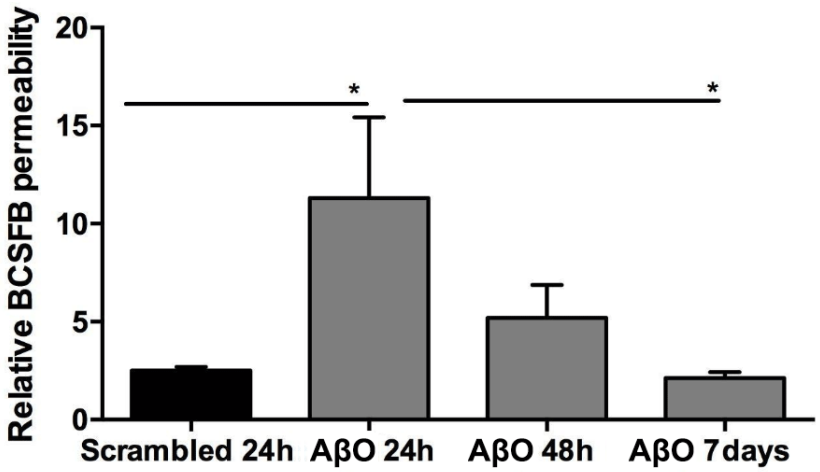

Figure 2. The prolonged effect of AβO i.c.v. injection on BCSFB integrity. Results are presented as relative ratio of FITC-dextran CSF concentration 24, 48 h and 7 days after i.c.v. AβO injection (grey) compared to scrambled peptide injection (black) .

4.1.3. The effects of i.c.v. injected AβO on CPE cell morphology

In order to test whether observed alterations in BCSFB permeability are coinciding with morphological changes of choroid plexus epithelial (CPE) cells, we used serial block-face scanning electron microscopy (SBF-SEM). Morphology of CPE cells was examined 6 h after injection either with AβO or scrambled control. The results represented in **Figure 3** show loss of the typical cuboidal shape of CPE cells after injection with AβO (**Figure 3b** and **3d**), while in animals injected with scrambled control, CPE cells retained their typical cuboidal morphology (**Figure 3a** and **3c**).

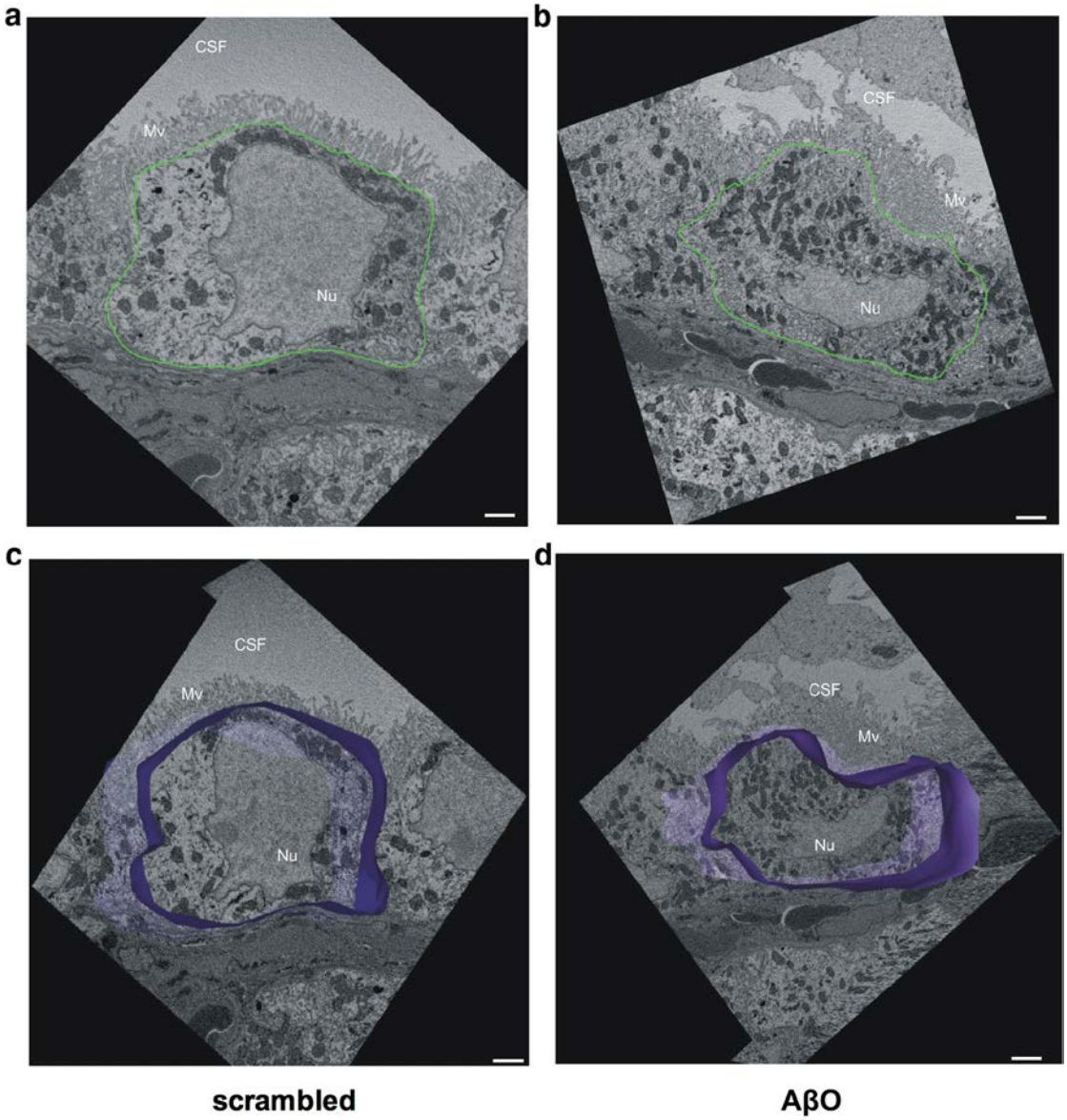

Figure 3. Choroid plexus epithelial (CPE) cell morphology analysis after AβO i.c.v. injection. Representative choroid plexus cells images of C57BL/6 mice i.c.v. injected with scrambled peptide (a) or AβO (b) at 6 h time point taken by serial block face scanning electron microscopy (SBF-SEM). Cell shape is outlined in green. 3D modeling (blue) based on merging ~200 sections of CPE cells from scrambled peptide (c) and AβO (d) injected mice. Only cell shape was considered, basolateral labyrinth and microvilli were neglected while generating the 3D modeling. CSF, cerebrospinal fluid; Mv, microvilli; Nu, nucleus; Scale bar = 2 μm.

4.1.4. The effect of i.c.v. injected AβO on tight junctions components expression in CPE cells

Upon observing the effect of AβO on the BCSFB permeability, it was investigated whether this was linked with changes in the expression of tight junctions (TJ) components. To address this, RT-qPCR and immunostainings were performed on CP tissue. As displayed in **Figure 4**, the obtained results revealed that mRNA expression for Claudin-1 (*Cldn1*), showed a significant decrease (31%, $p \leq 0.05$) 2 h after the injection of AβO in comparison to control (**Figure 4a**), while Claudin-5 (*Cldn5*) mRNA expression was significantly downregulated (68%, $p \leq 0.001$) at the 6 h time point (**Figure 4b**). Occludin (*Ocln*) mRNA expression was significantly decreased in both 2 h (36%, $p \leq 0.05$) and 6 h (38%, $p \leq 0.05$) after i.c.v. injection of AβO (**Figure 4c**). Since *Ocln* mRNA expression showed pronounced changes in both time points, immunostaining was done in order to visualize OCLN protein expression. The results revealed that OCLN is enriched on apical side of CPE cells in control animals i.c.v. injected with scrambled peptide (**Figure 4d**), while clear reduction in intensity of OCLN immunostaining was observed in animals i.c.v. injected with AβO (**Figure 4e**).

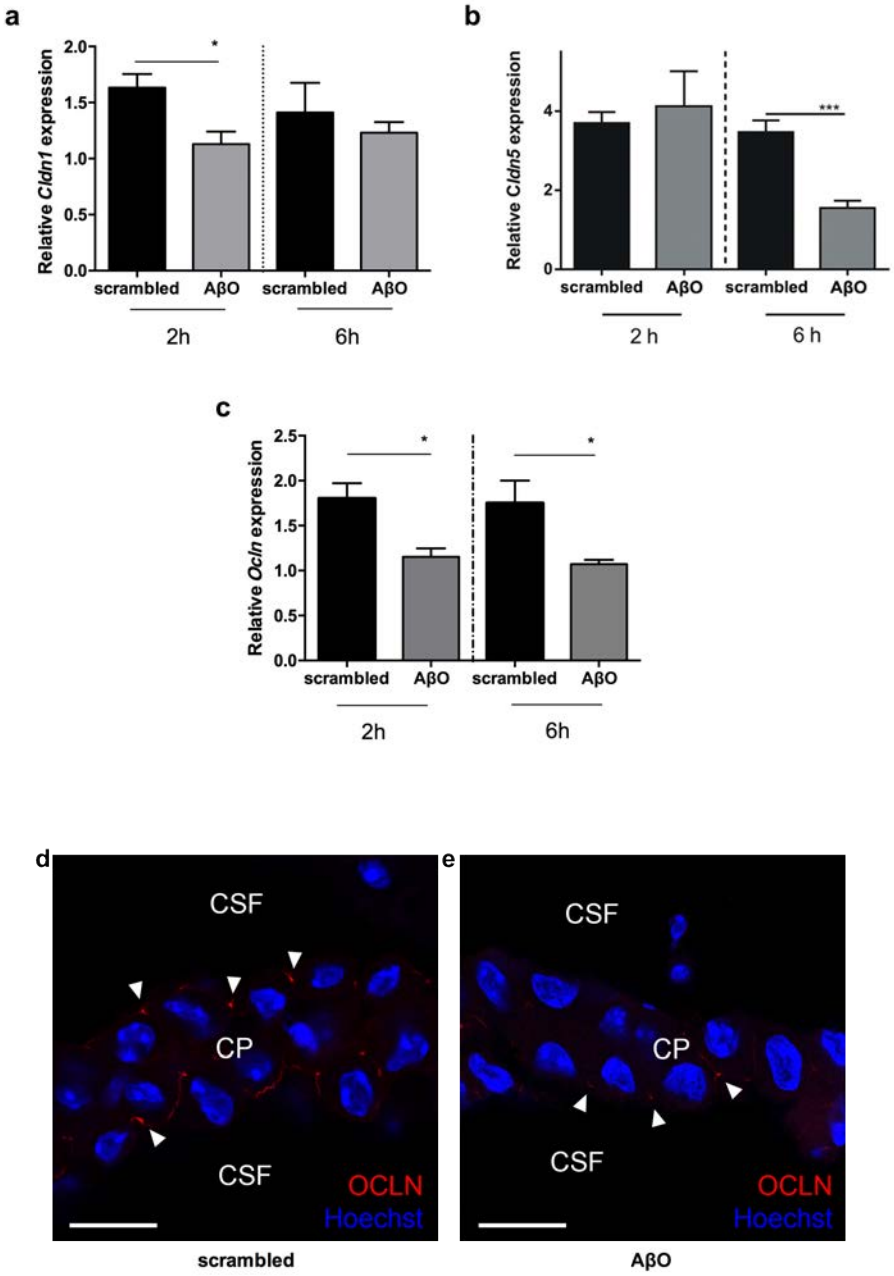

Figure 4. Expression of tight junctions components in CPE cells after AβO injection. Relative *Cldn1* (a), *Cldn5* (b) and *Ocln* (c) mRNA expression in the CP at 2 and 6 h time points after AβO i.c.v. injection (grey) compared to control injected with scrambled peptide (black). Representative choroid plexus cross section images of OCLN immunostaining in mice i.c.v. injected with scrambled peptide (d) or AβO (e) at 6 h time point (red, OCLN; blue, nuclei stained with Hoechst). The arrowheads point to the apically located tight junctions. CP, choroid plexus; CSF, cerebrospinal fluid; Scale bar = 10 μm.

Next, expression of zonula occludens 1 (ZO-1) gene expression was examined (**Figure 5**). *Zo1* mRNA expression also followed the trend of *Cldn1*, with notable downregulation (63%, p≤ 0.01) 2 h after i.c.v. injection with AβO. However, at 6 h after injection mRNA returned to control levels (**Figure 5a**). Immunostaining revealed decreased intensity of ZO-1 immunofluorescence in choroid plexus cross sections of animals i.c.v. injected with AβO (**Figure 5c**) in comparison to control animals i.c.v injected with scrambled peptide (**Figure 5b**).

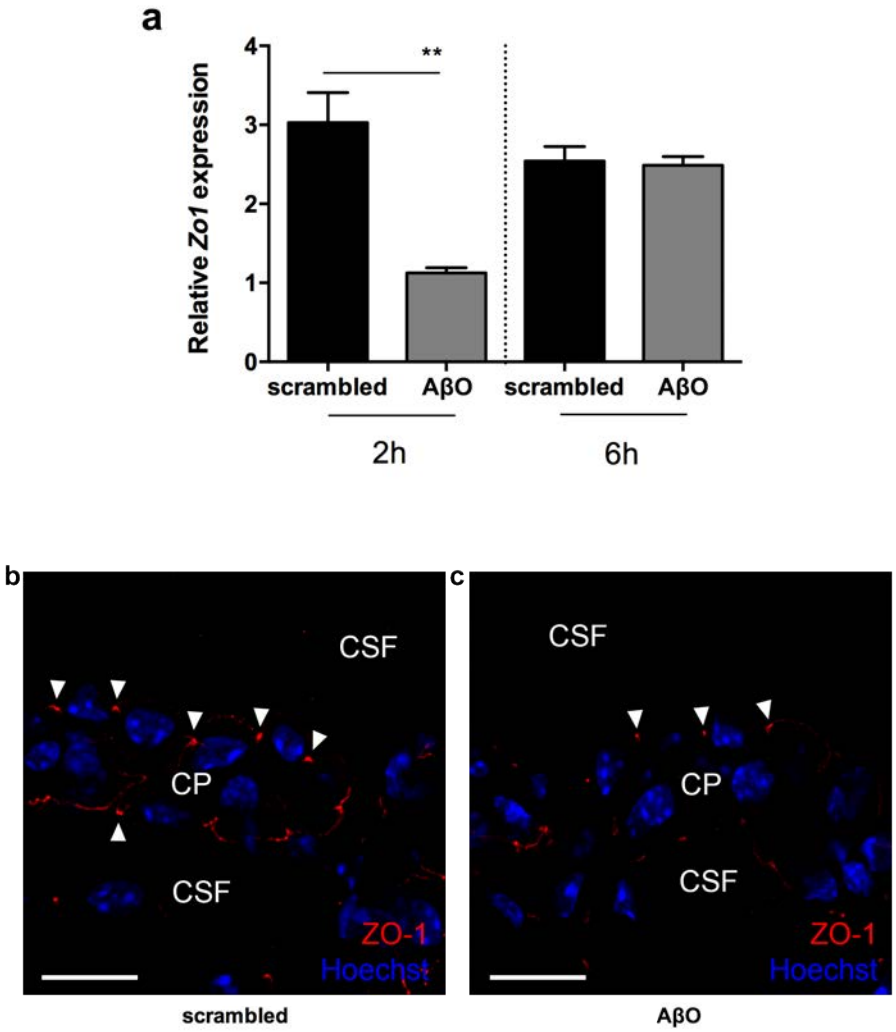

Figure 5. Expression of zonula occludens 1 in CP cells after AβO injection. Relative *Zo1* mRNA expression (a) 2 and 6 h after i.c.v. injection with AβO (black) or scrambled (grey) control. Representative choroid plexus cross section images of ZO-1 staining in mice i.c.v. injected with scrambled peptide (b) or AβO (c) at 6 h time point (red, ZO-1; blue, nuclei stained with Hoechst). The arrowheads point to the apically located tight junctions. CP, choroid plexus; CSF, cerebrospinal fluid; Scale bar = 10 μm.

4.1.4. The effect of i.c.v. injected AβO on gap and adherens junction components expression in CPE cells

In order to assess whether other junction complexes were affected by the compromised BCSFB integrity, mRNA expression analysis of gap and adherens junctions components was performed (**Figure 6**). Gene expression of gap junction component Connexin 43 (*Cx43*), as well as adherens junction components E-cadherin (*Cdh1*) and N-cadherin (*Ncdh*), was not significantly altered after AβO injection in comparison to control at both 2 and 6 h time points (**Figure 6a**, **6b** and **6c**). Moreover, ECDH immunostaining revealed that localization and integrity of adherens junctions were not changed in both AβO and scrambled peptide injected animals 6 h after injection (**Figure 6d** and **6e**).

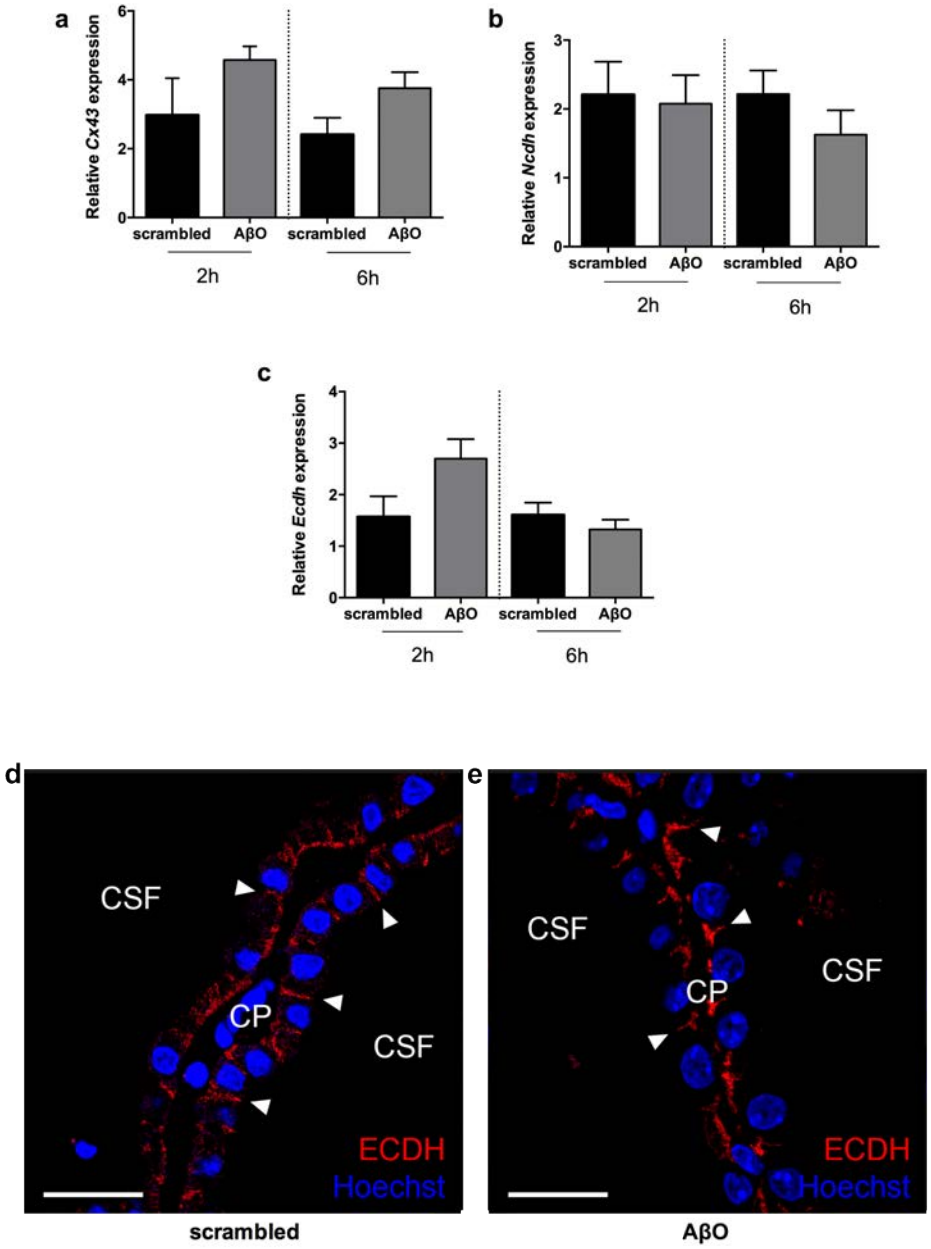

Figure 6. Expression of gap and adherens junction components after AβO injection. Relative mRNA expression of *Cx43* (a), *Ncdh* (b) and *Ecdh* (c) 2 and 6 h after AβO i.c.v. injection (grey) compared to scrambled peptide injection (black). Representative confocal choroid plexus cross section images of ECDH staining in mice i.c.v. injected with scrambled peptide (d) or AβO (e) at 6 h time point (red, ECDH; blue, nuclei stained with Hoechst). The arrowheads point to the tight junctions. CP, choroid plexus; CSF, cerebrospinal fluid; Scale bar = 10 μm.

4.2. The effect of i.c.v. injected AβO on blood-brain barrier (BBB) functionality

4.2.1. The effect of i.c.v. injected AβO on BBB integrity

Our next goal was to analyze if AβO affects the integrity of blood-brain barrier (BBB) in a similar manner as previously observed in BCSFB. To that end, BBB permeability was analyzed by measurement of 4 kDa FITC-dextran concentration in the brain tissue of the animals previously i.c.v. injected with AβO or scrambled peptide. The obtained data did not reveal any change in the permeability of BBB at the 6 h time point as displayed in **Figure 7**.

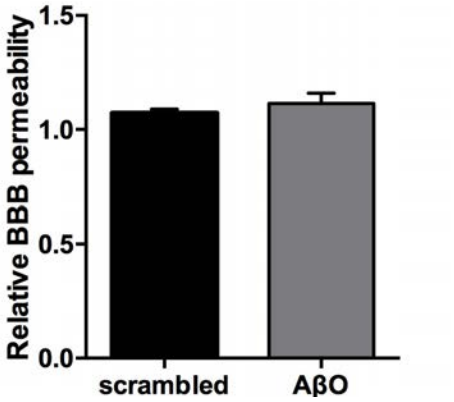

Figure 7. Analysis of BBB integrity after i.c.v. AβO injection. Results are presented as relative ratio of 4 kDa FITC-dextran concentration in CSF 6 h after AβO injection in the cerebral ventricles (grey) compared to scrambled peptide injection (black).

4.2.2. The effect of i.c.v. injected AβO on TJ components expression in BBB endothelial cells

Gene expression of tight junction components *Cldn1, Cldn5, Ocln* and *Zo1* was assessed using RT-qPCR. *Cldn1, Ocln* and *Zo1* gene expression remained unaltered 6 h after AβO injection in comparison to scrambled peptide control injection (**Figure 8a, 8c** and **8d**). In contrast, *Cldn5* mRNA expression was significantly decreased (2-fold, p≤0.01) at the same time point (**Figure 8b**).

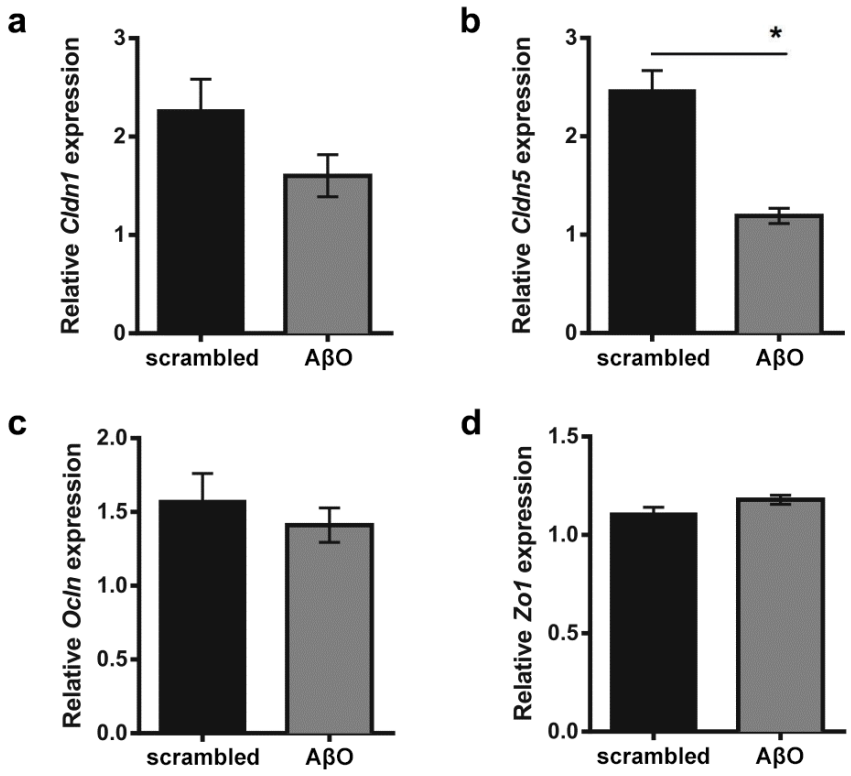

Figure 8. Expression of junctional complex components on endothelial cells of BBB. Relative mRNA expression of *Cldn1* (a), *Cldn5* (b), *Ocln* (c) and *Zo1* (d) 6 h after AβO (grey) or scrambled peptide (black) injection.

4.3. Effect of AβO i.c.v. injection on cognitive function

In order to inspect whether observed changes impact behavior of the animals, novel object recognition (NOR) test was performed. Short term and long term memory were assessed after AβO or scrambled peptide i.c.v. injection. Our findings revealed that in mice injected with AβO, short term memory showed decline ($p \leq 0.01$) (**Figure 9a**), as well as long term memory ($p \leq 0.01$) (**Figure 9b**).

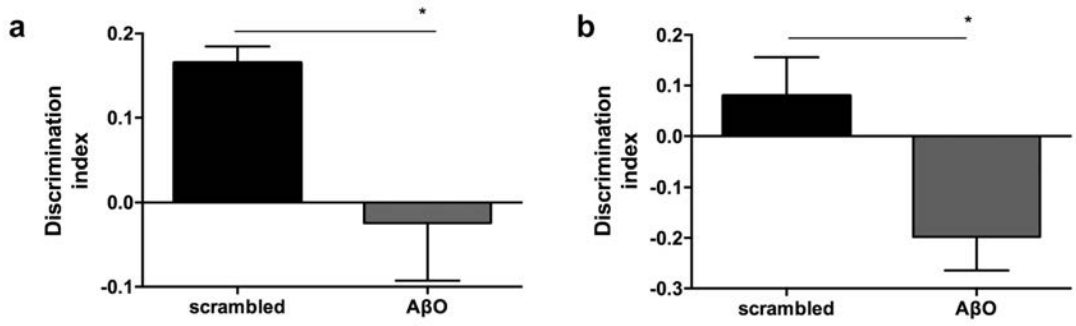

Figure 9. Behavioral changes after AβO i.c.v. injection. Short term memory assessment (a) and long term memory assessment (b) after AβO (grey) or scrambled peptide (black) injection.

4.4. The mechanism underlying the increase in BCSFB permeability induced by i.c.v. AβO injection

In order to investigate the mechanism underlying the observed BCSFB permeability increase following AβO injection the role of matrix metalloproteinases was analyzed further.

4.4.1. The effect of AβO i.c.v. injection on matrix metalloproteinases gene expression level in the CP

Gene expression analysis of several matrix metalloproteinases (MMPs), *Mmp3*, *Mmp8* and *Mmp9* in the CP tissue, using RT-qPCR, 6 h after scrambled peptide or AβO i.c.v. injection was performed (**Figure 10**). Most prominent increase in gene expression was observed on *Mmp3* mRNA level (12-fold, $p \leq 0.05$) (**Figure 10a**). Also, *Mmp8* and *Mmp9* mRNA expression exhibited a significant upregulation (3-fold, $p \leq 0.01$ (**Figure 10b**) and 2-fold, $p \leq 0.05$ (**Figure 10c**), respectively).

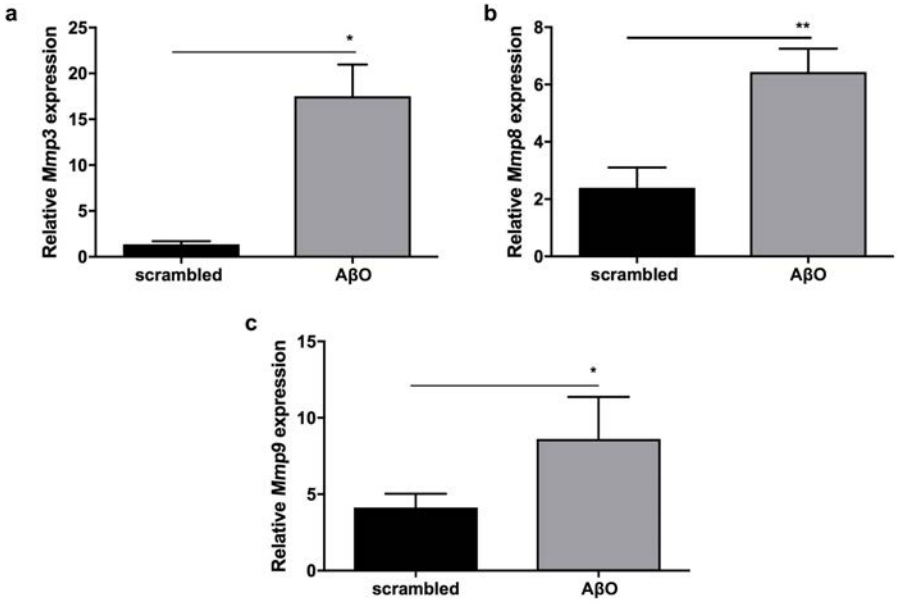

Figure 10. MMPs gene expression in CPE cells after AβO injection. Relative mRNA expression of *Mmp3* (a), *Mmp8* (b) and *Mmp9* (c) 6 h after AβO (grey) or scrambled (black) control i.c.v. injection.

4.4.2. The effect of AβO on MMP activity in CSF

MMP activity was analyzed in CSF 6 h after AβO or scrambled peptide injection by measuring fluorescence of the cleaved broad spectrum fluorogenic substrate for matrix metalloproteinases. The obtained results (**Figure 11**) show an increase in fluorescent signal which corresponds to elevated MMP activity in CSF of mice injected with AβO, in comparison to animals injected with scrambled peptide (6-fold, $p \leq 0.05$). Furthermore, injection of broad spectrum MMP inhibitor, GM6001, together with AβO showed decrease in the fluorescent signal, corresponding to diminished MMP activity (7-fold, $p \leq 0.01$).

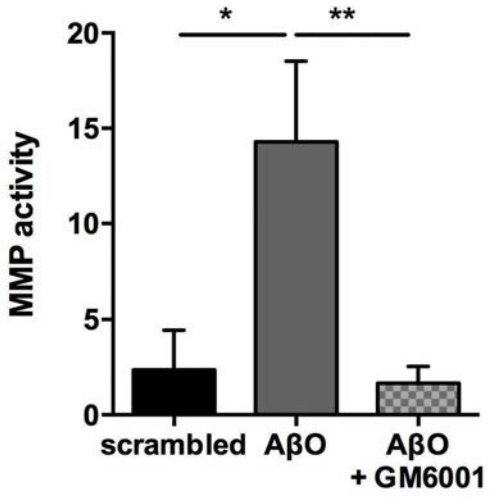

Figure 11. Analysis of MMP activity in CSF of mice i.c.v. injected with scrambled peptide, AβO or AβO combined with GM6001. MMP activity was analyzed 6 h after i.c.v. injection of scrambled peptide (black), AβO (grey) and GM6001 plus AβO (square pattern) in mice CSF.

4.4.3. The effect of MMP inhibition on AβO-induced increase in BCSFB permeability

As shown in **Figure 12**, injection of the broad spectrum MMP inhibitor GM6001, together with AβO, prevented the AβO-induced BCSFB permeability (59%, $p<0.0001$). However, in comparison to control animals, BCSFB permeability was still significantly increased (21%, $p \leq 0.01$).

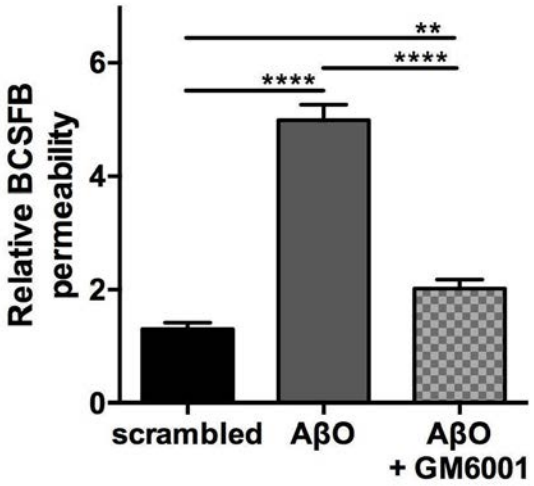

Figure 12. Relative BCSFB permeability after injection with scrambled peptide, AβO or mixture of AβO and GM6001. Results are presented as relative ratio of FITC-dextran concentration in CSF 6 h after scrambled peptide (black), AβO (grey) or mixture of AβO and GM6001 (square pattern) injection.

4.4.4. The effect of AβO on BCSFB permeability in MMP-3 deficient mice

As shown in **Figure 9a,** *Mmp3* gene expression was highly upregulated upon i.c.v. injection of AβO, and this was associated with increased MMP activity in the CSF (**Figure 13**). Next, the contribution of MMP-3 in AβO-induced increase in BCSFB permeability was examined. To address this, MMP-3 deficient mice were

used. To study BCSFB permeability, i.v. injection of 4 kDa FITC-dextran. BCSFB permeability was tested in MMP-3 deficient mice after AβO and scrambled peptide injection and compared to BCSFB permeability in wild type (C57BL/6) mice. As shown in **Figure 13**, following AβO injection a 7-fold increase in permeability in C57BL/6 mice was observed ($p \leq 0.01$). Interestingly, in comparison to their control animals, MMP-3 deficient AβO-injected mice still exhibited significant increase in permeability of BCSFB (89%, $p \leq 0.01$). Nevertheless, AβO-induced increase in BCSFB permeability was significantly, 3-fold lower in MMP-3 deficient compared to C57BL/6 mice ($p \leq 0.01$).

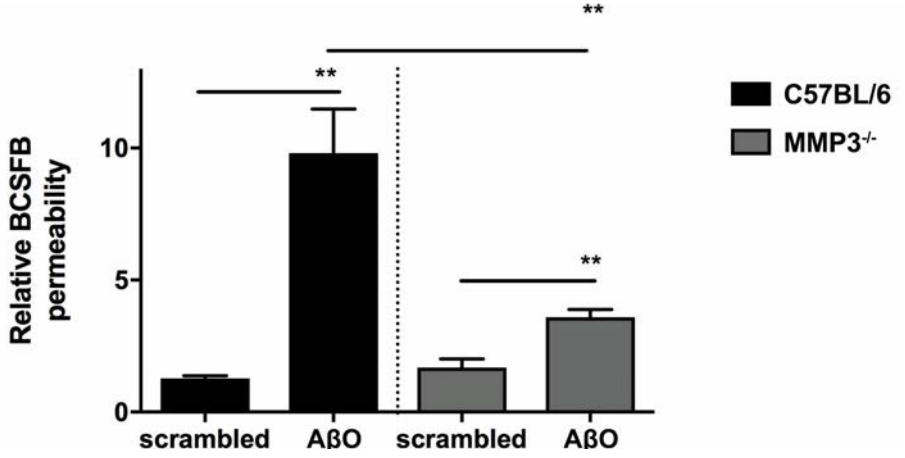

Figure 13. Relative BCSFB permeability after AβO injection in C57BL/6 and MMP3-/- mice. Results are presented as relative ratio of FITC-dextran CSF concentration 6 h after AβO injection in the cerebral ventricles compared to scrambled peptide injection, in both strains C57BL/6 (black) and MMP3-/- (grey)

4.5. The effect of i.c.v. AβO and intraperitoneal LPS injection on secretory activity of CP

4.5.1. The effect of i.c.v. injected AβO on transthyretin (TTR) expression in CPE cells

Transthyretin (*Ttr*) mRNA and protein expression was analyzed using RT-qPCR and immunostaining comparing mice injected with scrambled peptide or AβO. The results depicted in **Figure 14** show that i.c.v. AβO injection did not affect *Ttr* mRNA expression 2 h and 6 h after injection in the CP (**Figure 14a**). Additionally, confocal imaging of sections stained with fluorescently labeled TTR antibody did not show any significant alterations in TTR protein expression 6 h after AβO injection compared to control (**Figure 14b** and **14c**).

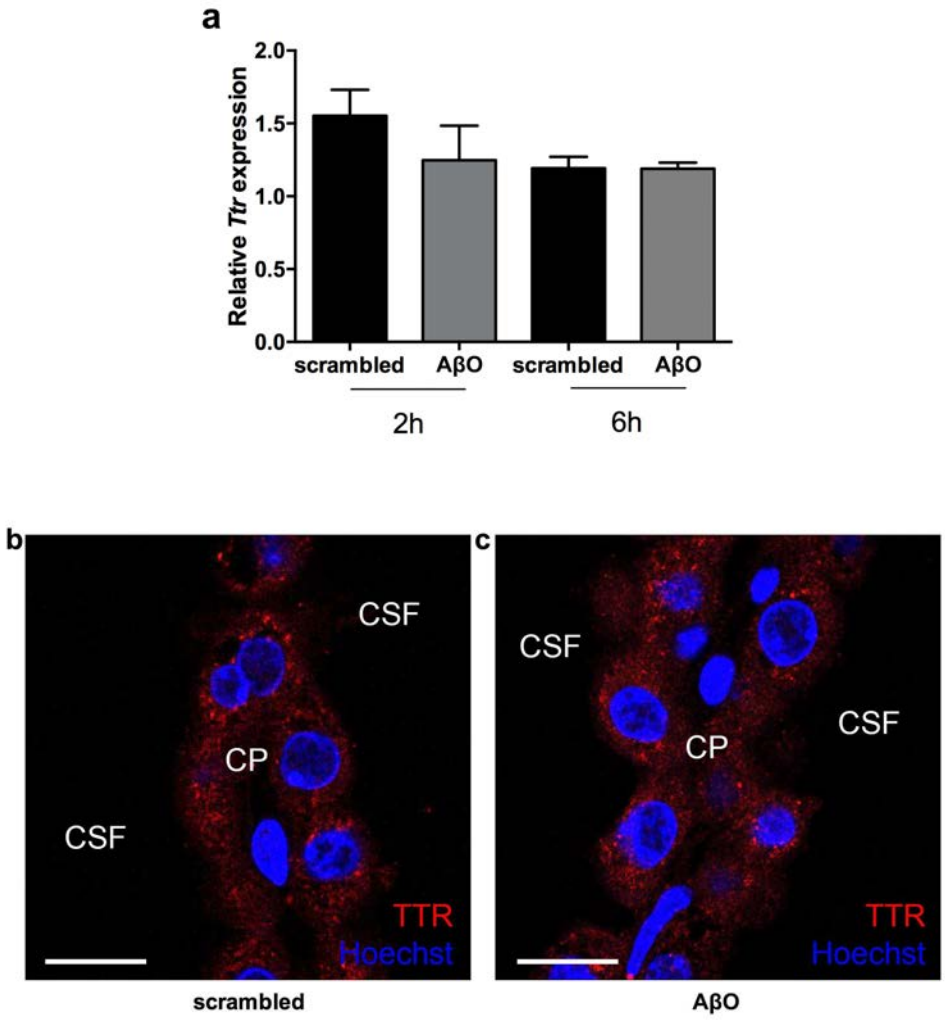

Figure 14. Expression of transthyretin after AβO injection. Relative mRNA expression of *Ttr* (a), 2 and 6 h after i.c.v. AβO injection (grey) compared to scrambled peptide injection (black). Representative confocal cross section images of TTR staining in choroid plexus of mice i.c.v. injected with scrambled peptide (b) or AβO (c) at 6 h time point (red, TTR; blue, nuclei stained with Hoechst). CP, choroid plexus; CSF, cerebrospinal fluid; Scale bar = 10 μm.

4.5.2. The effect of i.c.v. injected AβO on brain derived neurotrophic factor expression in the CP and CSF

Gene and protein expression of brain derived neurotrophic factor (BDNF) was assessed using RT-qPCR and ELISA, respectively, and presented in **Figure 15**. RT-qPCR analysis demonstrated that 2 h after i.c.v. AβO injection *Bdnf* expression

in the CP was significantly downregulated (43%, p≤ 0.05) (**Figure 15a**). In agreement with this, ELISA assay showed a significant drop in BDNF levels in CSF of AβO injected animals, in comparison to scrambled peptide injected animals (79.61%, p≤ 0.01) (**Figure 15b**).

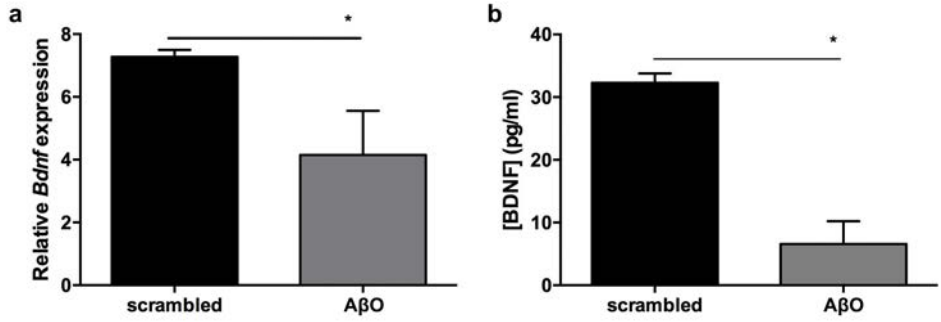

Figure 15. *Bdnf* mRNA expression in the CP and BDNF protein concentration in CSF after AβO injection. Gene expression analysis of *Bdnf* in CPE cells 2 h after AβO (grey) or scrambled (black) i.c.v. injection (a). Concentration of BDNF in CSF 6 h after AβO (grey) or scrambled (black) i.c.v. injection (b).

4.5.3. The effect of i.c.v. injected AβO on expression of inflammatory mediators in the CP and CSF

RT-qPCR analysis showed a significant increase in mRNA expression of all three examined proinflammatory cytokines at both time points in AβO injected mice, in comparison to control mice (**Figure 16**). *Il1β* mRNA level was increased 3-fold (p≤ 0.001) at the 2 h time point and 7-fold (p≤ 0.05) at the 6 h time point (**Figure 16a**). *Il-6* mRNA level was increased 10-fold (p≤ 0.05) at 2 h time point and 48-fold (p≤ 0.01) at 6 h time point (**Figure 16b**). Similar, *Tnf* mRNA level was increased 9-fold (p≤ 0.01) at 2 h time point and 30-fold (p≤ 0.001) at 6 h time point (**Figure 16c**).

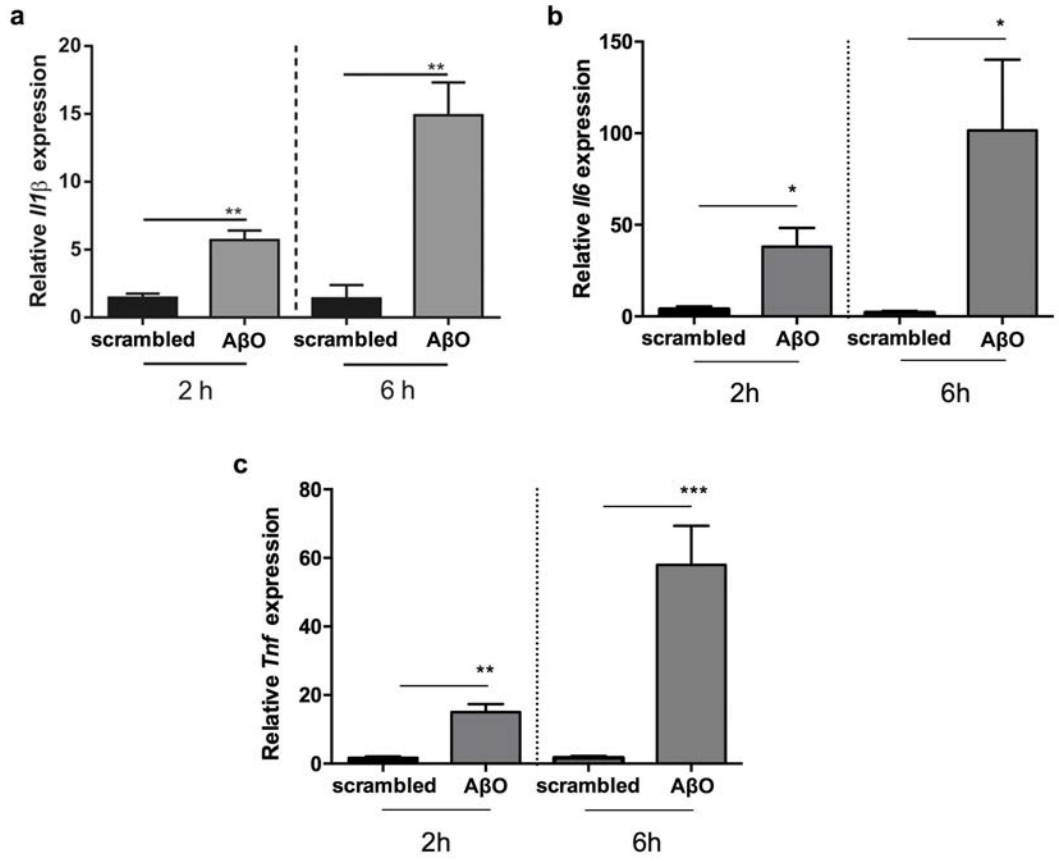

Figure 16. Gene expression of proinflammatory cytokines in the CP 2 and 6 h after AβO injection. Relative mRNA expression of *Il1β* (a), *Il6* (b) and *Tnf* (c) 2 and 6 h after AβO (grey) or scrambled peptide (black) i.c.v. injection.

Using the Bio-Plex® multiplex immunoassay system, significant increase in CSF protein levels of several interleukins was noted in AβO i.c.v. injected mice in comparison to scrambled control (**Figure 17**). IL-1α levels were significantly increased 2 h (34%, $p \leq 0.05$), and 2-fold ($p \leq 0.001$) 6 h upon i.c.v. AβO injection (**Figure 16a**). IL-1β levels were significantly increased already at 2 h (29%, $p \leq 0.05$) and elevation was 6-fold ($p \leq 0.01$) 6 h after i.c.v. AβO injection (**Figure 17b**). IL-3 was significantly increased (4-fold; $p \leq 0.05$) only at the 6 h time point (**Figure 17c**). Among the different cytokines that were analyzed in the CSF, IL-6 level was highest, with a 14-fold ($p \leq 0.05$) increase 2 h, and 41-fold ($p \leq 0.001$) increase 6 h after i.c.v. injection of AβO (**Figure 17d**). IL-10 was notably increased both 2 h (14%, $p \leq 0.05$) and 6 h (28%, $p \leq 0.001$) after injection (**Figure 17e**). IL-12p40 was

15-fold ($p \leq 0.05$) and 6-fold ($p \leq 0.05$) increased at 2 and 6 h upon i.c.v. injection of AβO injection, respectively (**Figure 17f**). IL-13 was 2-fold ($p \leq 0.05$) increased 2 h and 3-fold ($p \leq 0.001$) 6 h after injection (**Figure 17g**). IL-17 could not be detected in scrambled control injection mice, but showed a 50-fold ($p \leq 0.01$) increase 6 h after i.c.v. injection of AβO (**Figure 17h**).

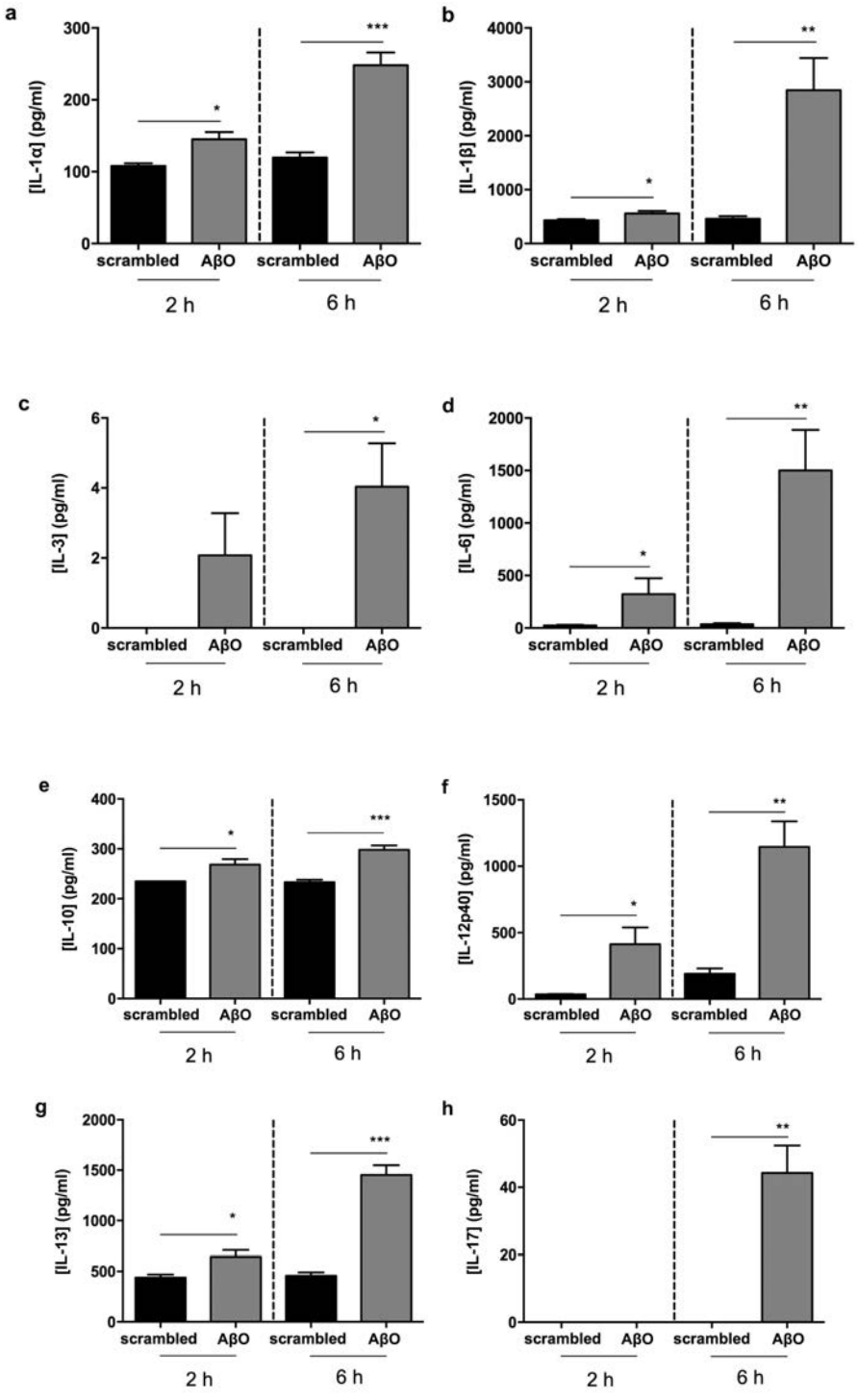

Figure 17. Interleukin protein levels in CSF upon AβO injection. Concentration (pg/ml) of IL-1α (a), IL-1β (b), IL-3 (c), IL-6 (d), IL-10 (e), IL-12p40 (f), IL-13 (g) and IL-17 (h) in CSF 2 and 6 h after AβO (grey) or scrambled peptide i.c.v. injection (black).

Using the same method, it was determined that several chemokine levels were significantly increased in CSF 2 and 6 h after AβO injection, in comparison to control (**Figure 18**).

Rantes CSF protein levels remained unaltered 2 h after injection, while 31-fold ($p \leq 0.05$) increase was noted 6 h after i.c.v. injection (**Figure 18a**). MCP-1 was 18-fold ($p \leq 0.001$) and 2-fold ($p \leq 0.01$) increased at 2 and 6 h time points, respectively (**Figure 18b**). MIP-1α was 2-fold ($p \leq 0.01$) increased at 2 h time point, and 5-fold ($p \leq 0.001$) increased at 6 h time point (**Figure 18c**). MIP-1β was 5-fold ($p \leq 0.01$) increased at 2 h and 7-fold ($p \leq 0.001$) increased at 6 h time point (**Figure 18d**).

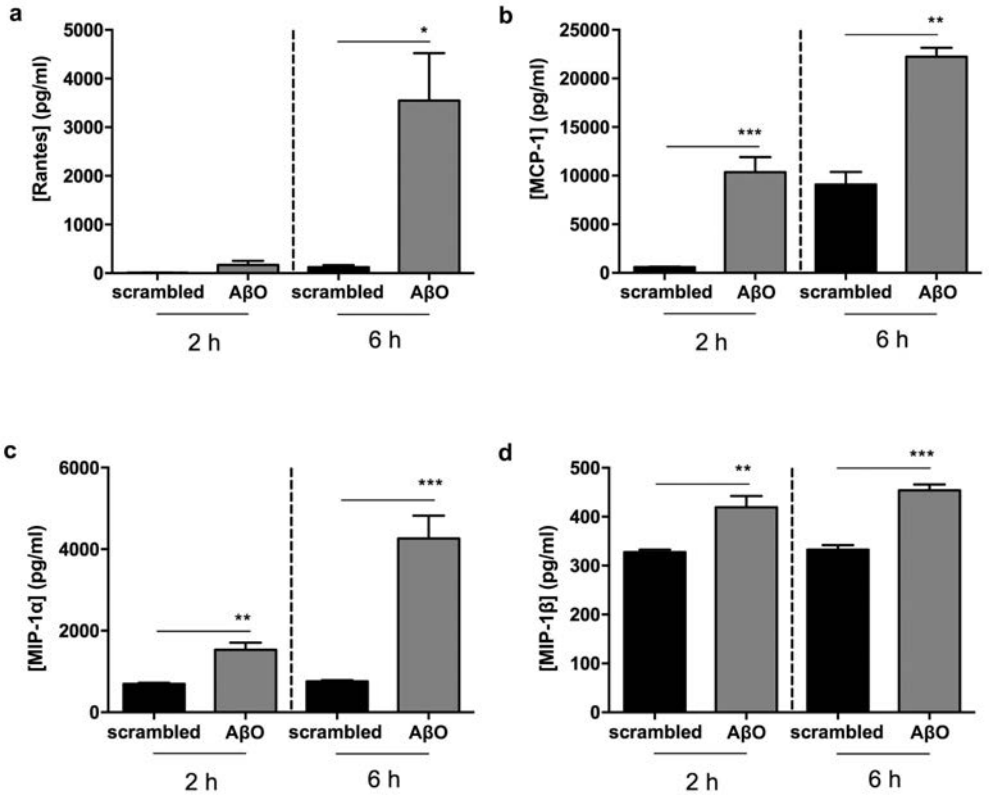

Figure 18. Chemokine levels in CSF upon AβO injection. Concentration (pg/ml) of Rantes (a), MCP-1 (b), MIP-1α (c) and MIP-1β (d) in CSF 2 and 6 h after AβO (grey) or scrambled peptide i.c.v. injection (black).

Several important proinflammatory cytokines from other families, such as colony stimulating family (GM-CSF), tumor necrosis factor family (TNF), interferon γ (IFN-γ) were tested using the same method and this revealed a significant increase in AβO injected group in comparison to scrambled peptide injected mice (**Figure 19**). GM-CSF was 700-fold ($p \leq 0.01$) increased at 2 h time point and 900-fold ($p \leq 0.05$) increased at 6 h time point (**Figure 19a**). IFN-γ was significantly, 6-fold ($p \leq 0.01$) increased only at the 6 h time point (F**igure 19b**). Also TNF was significantly increased at the 6 h time point (36%, $p \leq 0.001$) (**Figure 19c**).

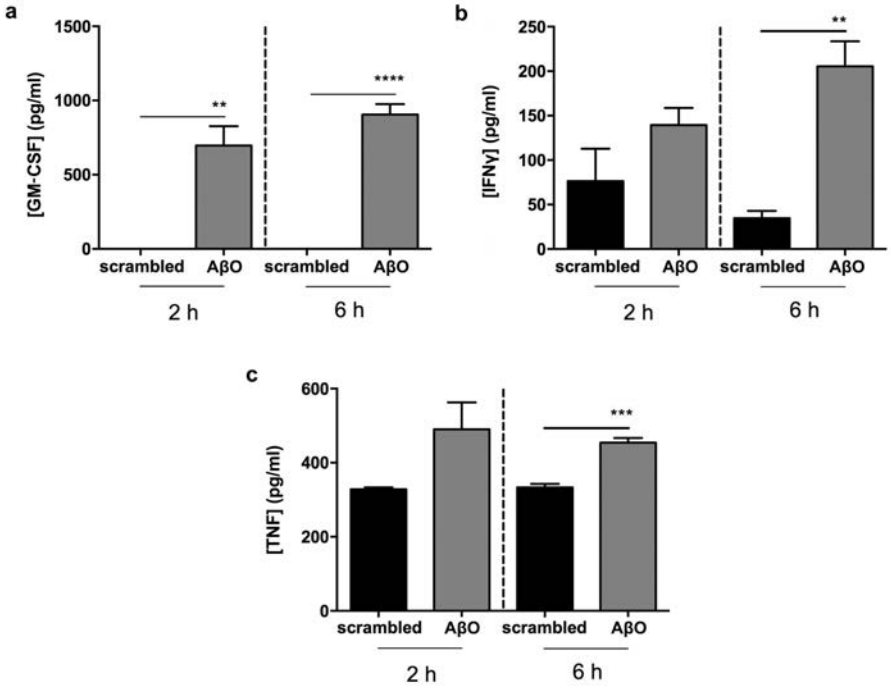

Figure 19. Cytokine levels in CSF after AβO injection. Concentration (pg/ml) of GM-CSF (a), IFN-γ (b) and TNF (c) in CSF 2 and 6 h after i.c.v. AβO (grey) or scrambled control injection (black).

4.5.4. The effect of i.c.v. injected AβO on expression of inflammatory mediators in the hippocampus

Next, in order to examine whether inflammatory response is notable in the rest of the brain, mRNA expression levels of the *Il1β*, *Il6* and *Tnf* were tested in hippocampus. *Il1β* was significantly increased at both, 2 (15-fold, p≤ 0.01) and 6 h (16-fold, p≤ 0.01) time points (**Figure 20a**), while *Il6* was only significantly upregulated at 2 h (2-fold, p≤ 0.001) (**Figure 20b**). *Tnf* was also found to be significantly increased at both 2 (9-fold, p≤ 0.001) and 6 h (11-fold, p≤ 0.01) time points (**Figure 20c**).

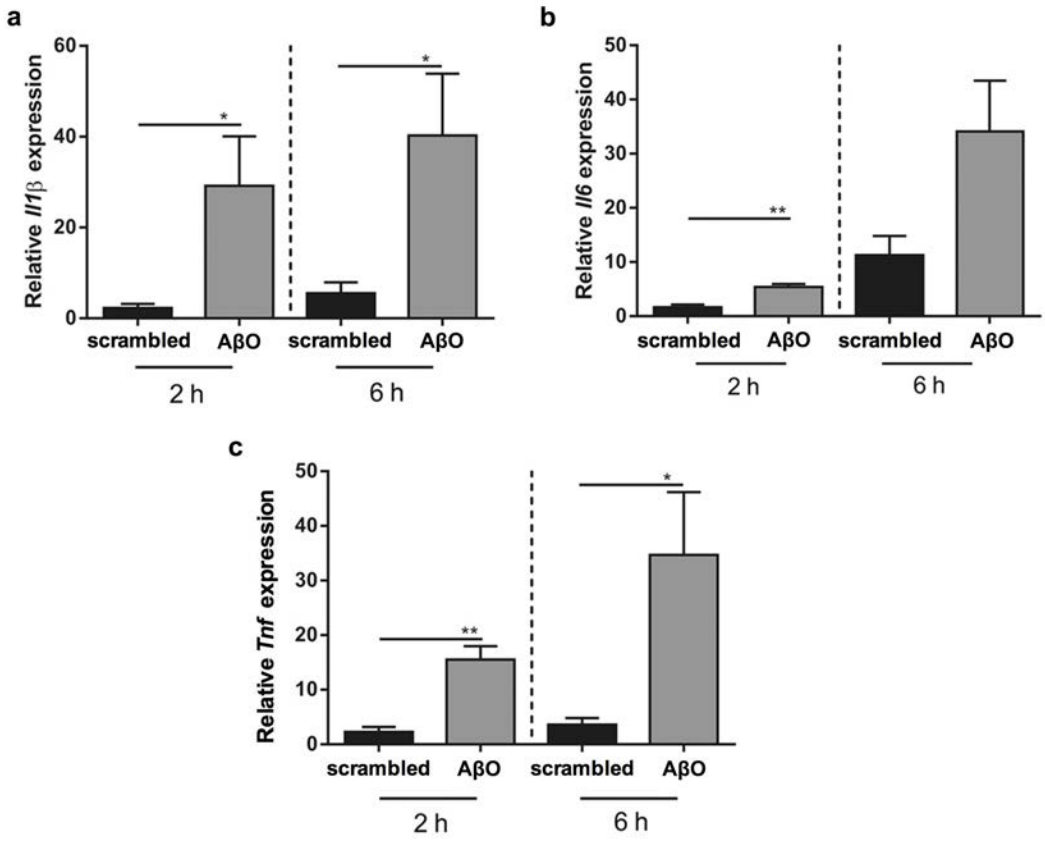

Figure 20. Gene expression of proinflammatory cytokines in hippocampus 2 and 6 h after AβO injection. Relative mRNA expression of *Il1β* (a), *Il6* (b) and *Tnf* (c) 2 and 6 h after AβO (grey) or scrambled peptide (black) i.c.v. injection.

4.6. The effect of an inflammatory stimulus on the number of particles in the CSF

4.6.1. The effect of i.c.v. injected AβO on the number of particles in CSF

In order to assess the number of particles, including extracellular vesicles (EVs) in CSF, nanoparticle tracking analysis (NTA) was performed. Particle size/distribution gained from NTA, showed that most of observed particles are in the range of 80-140 nm (data not shown). Representative video frame from NTA shows particles in CSF of scramble peptide (**Figure 21a**) and AβO (**Figure 21b**) i.c.v. injected mice. The results showed no alteration in the number of EVs in CSF 2 h after injection with AβO in comparison to injected scrambled peptide. Nevertheless, 6 h upon i.c.v. injection of AβO, a significant increase in number of EVs was observed in comparison to scrambled injected group at the same time point (3-fold increase, p≤0.001) **(Figure 21c)**.

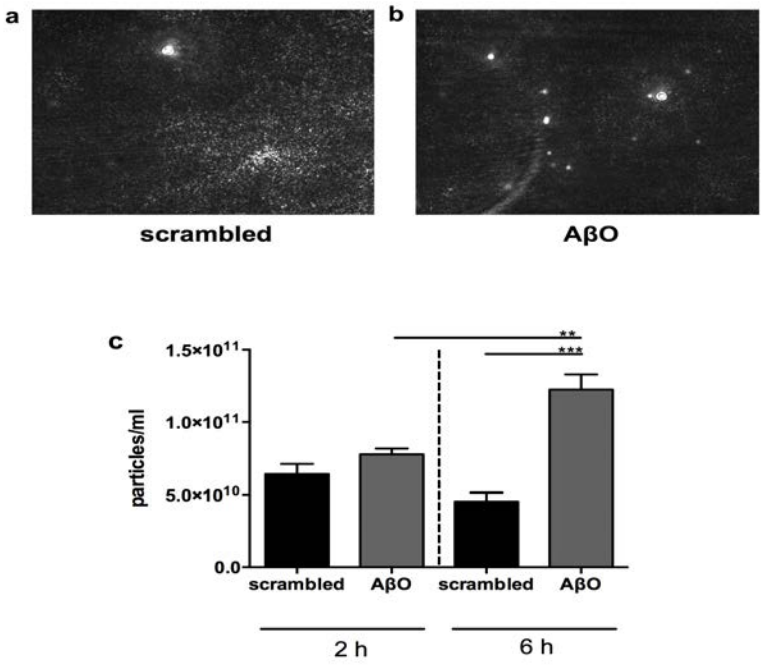

Figure 21. Amount of particles per ml in CSF 2 and 6 h after AβO injection. Representative video frame of particles in CSF of scrambled (a) or AβO (b) injected mice as observed on NTA Number of particles, including EVs, per ml in CSF after

AβO (grey) or scrambled peptide (black) injection in the cerebral ventricles of mice (d).

Later time point analysis revealed that the number of EVs in CSF were significantly increased at 24 h (4-fold, p≤0.001) after AβO i.c.v. injection compared to scrambled control. However, at 48 h and 7 days after AβO i.c.v. injection the number of vesicles significantly decreased compared to 24h time point (3 fold, p≤0.001 and 4-fold, p≤0.001 respectively) and reached the control level detected in scrambled injected animals (**Figure 22**).

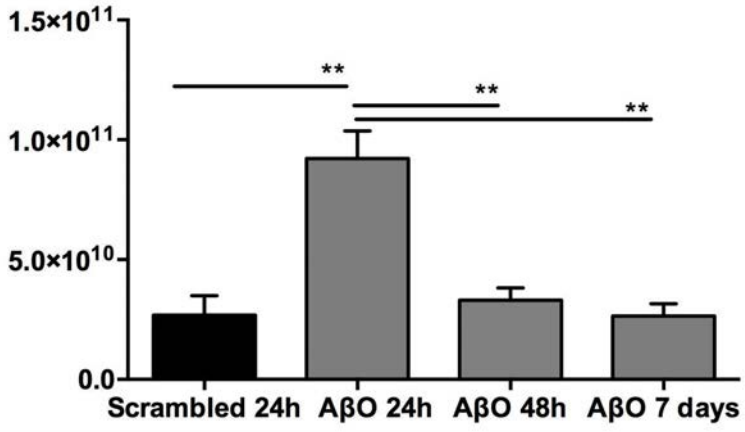

Figure 22. Amount of particles in CSF in later time points after AβO injection. Number of particles, including EVs, per ml in CSF, 24 h, 48 h and 7 days upon AβO (grey) or scrambled peptide (black) i.c.v. injection in mice.

4.6.2. The effect of i.p. injected LPS on the number of particles in CSF

To determine whether the number of particles, including EVs, was increased in CSF of LPS i.p. injected mice, the NTA technology was used. Representative video frame from NTA shows particles in CSF of control (**Figure 23a**) and LPS (**Figure 23b**) i.p. injected mice. As represented in **Figure 23c**, the results revealed a significant increase in the number of particles, including EVs,

detected in the CSF 2 h after i.p. LPS injection (89%, p≤0.05). This LPS effect was even more pronounced 6 h after i.c.v. injection (3 fold, p≤0.01).

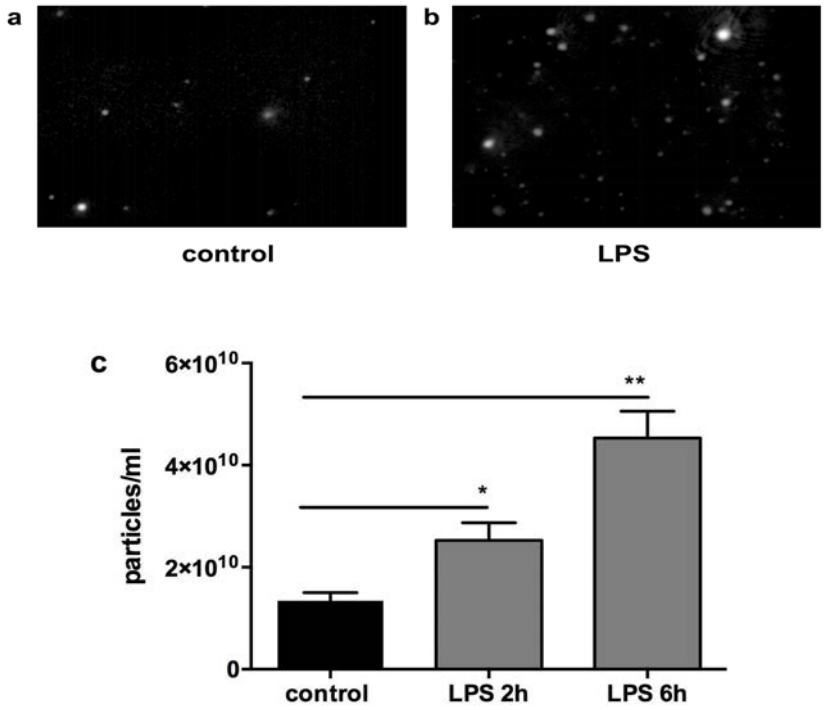

Figure 23. Measurement of the amount of particles in CSF of wild type mice injected i.p. with LPS. Representative images of particles in CSF of control (a) or LPS injected mice (b) as observed on NTA. Number of particles (per ml of CSF), including EVs, per ml in CSF after LPS (grey) or scrambled control (black) injection (c).

4.7. The effect of inflammatory stimulus on the expression of EVs marker and miRNA in the CP

4.7.1. The effect of AβO injection on CD63 expression

To determine whether the observed increase in EVs was driven by altered gene expression in the CP, the effect of AβO i.c.v. injection on the expression of CD63, a marker of EVs, was analyzed. For this purpose, RT-qPCR was performed on CP tissue. **Figure 24** shows that AβO-injected animals showed a significant increase of *Cd63* gene expression in CP (38%, $p \leq 0.01$).

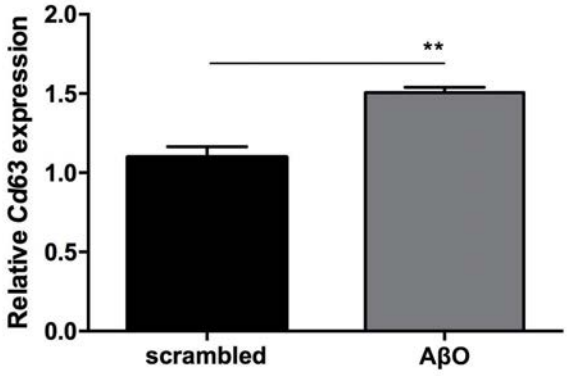

Figure 24. Gene expression of *Cd63* EV marker after AβO injection. Relative mRNA expression level of *Cd63* 6 h after AβO (grey) and scrambled control (black) i.c.v. injections.

4.7.2. The effect of i.p. LPS injection on CD63 expression

Next, gene expression levels of *Cd63* was measured in animals i.p. injected with LPS, and the findings are presented in **Figure 25**. Obtained results showed that 6 h after i.p. injection of LPS there was a significant induction of *Cd63* gene expression (5 fold, $p \leq 0.05$) in comparison to control animals.

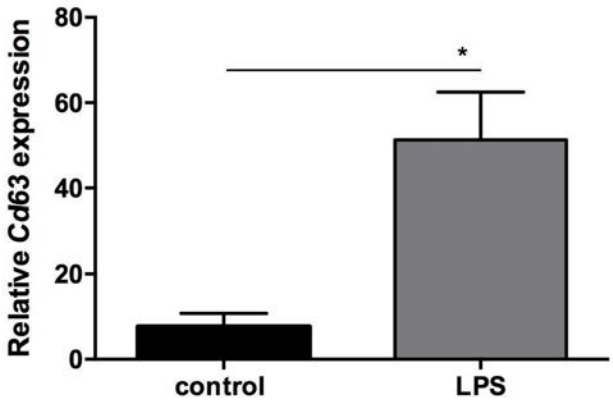

Figure 25. Gene expression of *Cd63* EV marker upon LPS injection. Relative mRNA expression of *Cd63* in control (black) and 6 h after i.p. LPS injection (grey).

4.7.3. The effect of i.c.v. injected AβO on miRNA expression in the CP

Using RT-qPCR, gene expression of this miRNA in CP, 6 h after i.c.v. AβO injection was tested. **Figure 26** shows that miR-155 levels were significantly three-fold increased($p \leq 0.05$).

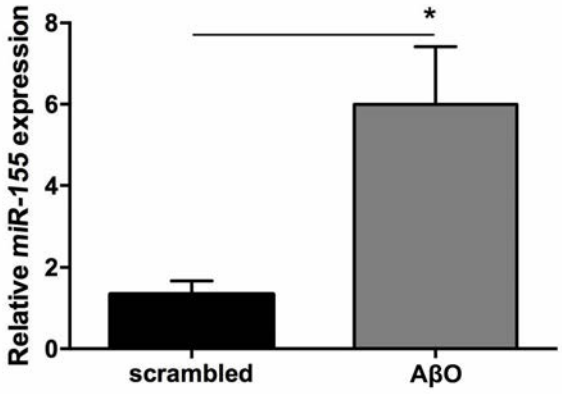

Figure 26. Expression analysis of miR-155 in CPE cells 6 h after AβO injection. Relative miR-155 expression 6 h after i.c.v. AβO (grey) or scrambled (black) control injection.

4.7.4. The effect of LPS injection on miRNA levels in the CP

Gene expression of miR-155 in CP was tested in the same manner as for i.c.v. AβO injection experiments. As observed in **Figure 27**, the same pattern of miR-155 expression as in i.c.v. AβO injection experiments was detected upon LPS treatment, displaying significant upregulation 6 h after i.p. LPS injection (7 fold, p≤ 0.01).

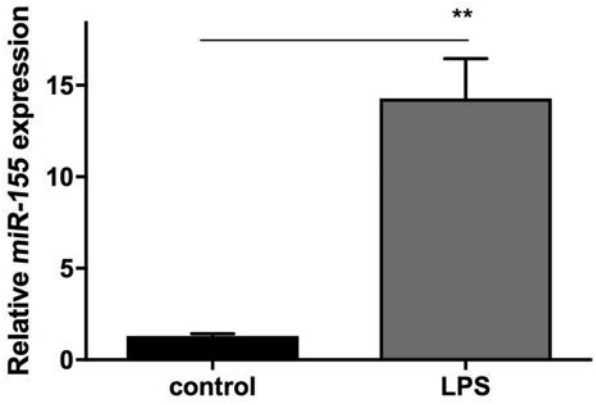

Figure 27. Gene expression of miR-155 in CP 6 h after i.p. LPS injection. Relative miRNA expression of miR-155 in control and 6 h after i.p. LPS injection.

4.8. Effect of AβO injection on microglial activation

Finally, the impact of AβO on the microglia was analyzed. Therefore, samples were immunostained with Iba1, a specific marker for both resting and activated microglial cells, and the microglia in the vicinity of the lateral ventricles 6 h after AβO or scrambled peptide i.c.v. injection were studied in more detail. Iba1-positive cells were counted and classified into resting and activated. The results depicted in **Figure 28** did not reveal a significant increase in the number of activated microglial cells at this time point in comparison with scrambled injected mice.

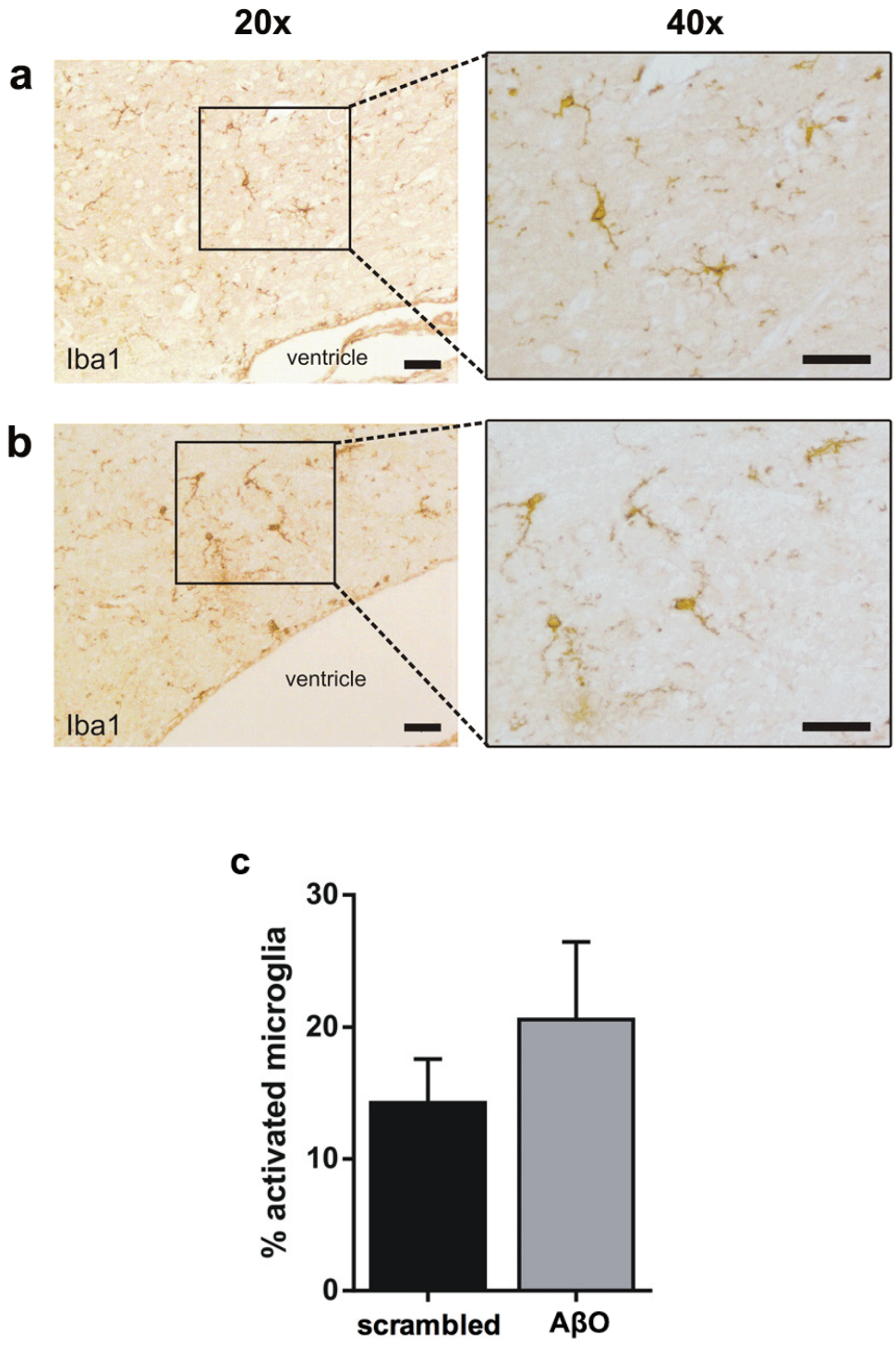

Figure 28. Microglial activation in the brain 6 h after AβO injection. Representative bright field images of Iba1 immunostaining in microglial cells in the vicinity of lateral ventricle of mice i.c.v. injected with scrambled (a) or AβO (b) at 6 h time point. Scale bar = 25 μm. Quantified percentage of activated microglia in total number of microglial cells observed in control and AβO injected animals (c).

5. Discussion

Inflammation is characterized by a physiological response of an organism to a harmful insult, occurring with the aim to elicit repair of the damaged tissue. However, if this inflammatory response remains unresolved and prolonged, it leads to a pathological condition, finally resulting in cell death. Neuroinflammation has been considered to be a common ground and crucial player in neurodegeneration observed in various central nervous system (CNS) disorders, such as traumatic brain injury, stroke, neurodegenerative diseases, multiple sclerosis (MS), and various infections (Schwartz and Baruch, 2014). The primary inflammatory response is often not the cause of tissue damage. It is rather the secondary injury, which is the result of an uncontrolled inflammatory response, that inflicts a greater harm.

In all above mentioned disorders, a similar pattern is observed regarding the series of events occurring in the brain: local production of inflammatory mediators, opening of the CNS barriers, trafficking of leukocytes, and activation of microglial cells (Brkic et al., 2015a). Microglia are believed to be the key players in exacerbating the inflammatory response (Block et al., 2007). In contrast, it has been shown that microglial cells are not sufficient for CNS repair and recovery, and leukocytes from the systemic circulation are also involved in these processes (Moalem et al., 1999). Consequently, CNS barriers play an important role in neuroinflammation, and novel data show that they are dynamic structures, actively involved in entry of immune cells and the regulation of the inflammatory response in the brain. Until several years ago, most attention has been given to the blood-brain barrier (BBB), since it is the largest of all CNS barriers. However, recently a pivotal role in leukocyte trafficking was assigned to the blood-cerebrospinal fluid barrier (BCSFB), an important part of the choroid plexus (CP). However, lack of the adequate models to study the BCSFB and the CP delayed research efforts in this field (Lopes Pinheiro et al., 2016). Due to its specific localization in all four ventricles, its unique structure of a tight epithelial barrier that surrounds fenestrated capillaries, the presence of resident inflammatory cells in its stroma, important characteristics such as a major production site of CSF, and presence of numerous transporters and receptors which influence brain homeostasis and

functioning, the **CP has been lately recognized as immune surveillance organ of the brain and important relay station between the periphery and CNS in inflammatory processes** (Kant et al., 2018).

To test the role of the CP in the initiation of neuroinflammation and uniformity of its response across several diseases, two animal models of conditions associated with neuroinflammation were used. For this thesis, research was performed using one of the available mouse models of sepsis, namely intraperitoneal (i.p.) injection of lipopolysaccharide (LPS), a component of the Gram negative bacterial cell wall. In this model we observerd changes in several main physiological characteristics of the CP, including increased BCSFB permeability, altered choroid plexus epithelial (CPE) cell morphology and increased secretory activity in the CP. In addition, the same model was used to further examine CP-brain communication during initiation of inflammation via the secretion of extracellular vesicles (EVs). Moreover, since studies that investigate the role of the CP in Alzheimer's disease (AD) are currently limited, some of the alterations seen in the LPS model, were studied in a recently developed mouse model of AD, namely intracerebroventricular (i.c.v.) injection of Aβ 1-42 oligomers (AβO) model. Finally, to be able to examine uniformity of the response of CP in the initiation of neuroinflammation, the results were compared with the findings from literature on other conditions associated with neuroinflammation, such as multiple sclerosis, traumatic brain injury and stroke.

A common mechanisms in various conditions associated with neuroinflammation is the entrance of immune cells into the brain via the BCSFB (Kaur et al., 2016). In physiological conditions, the sealing of tight junctions (TJs) between adjacent CPE cells restricts the passage of undesired pathogens, cells and molecules. Thus, to allow paracellular passage of leukocytes, changes in CPE cell morphology and downregulation or delocalization of components of junctional complexes often occur early in the neuroinflammatory processes.

The host lab previously showed that already 4 h after i.p. LPS injection an increase in BCSFB permeability can be detected, associated with morphological

changes of CPE cells from cuboidal to round observed 8 h after the injection (Vandenbroucke et al., 2012). Interestingly, nowadays LPS i.p. injection is perceived as a common method to create pro-inflammatory environment in the CNS (Catorce and Gevorkian, 2016).

In this thesis, the impact of an inflammatory stimulus originating from the CNS on the BCSFB was analyzed. This revealed similar effects compared to systemic inflammation. Indeed, already 6 h after i.c.v. injection of AβO, an increase in BCSFB permeability was observed. This finding was accompanied with morphological changes of the CPE cells from their characteristic cuboidal shape to more flattened morphology. Additionally, downregulation of several genes encoding TJ components, *e.g.* Claudin-1 (*Cldn-1)*, Claudin-5 (*Cldn-5)*, Occludin (*Ocln)* and Zonula occludens-1 (*Zo-1)* was noted. Also, using immunohistochemical stainings, reduced OCLN signal at the apical side of the cell, where it is normally positioned, was seen. The research performed for this thesis, also showed that adherens and gap junctional complexes were not affected. Moreover, this study revealed that BCSFB permeability returned to control levels already 7 days after i.c.v. AβO injection (Brkic et al., 2015b).

In the literature, similar CP responses have been observed in various diseases associated with neuroinflammation, regardless of whether stimuli were from peripheral or CNS origin. In brain diseases caused by infection, *e.g.* in a rodent model of acute ventriculitis, severe changes in CPE cells were observed. This was reflected by loss of cuboidal structure of the CPE cells, reduced number of microvilli, and a flattened epithelial surface (Cardia et al., 1995). Similarly, CPE morphological abnormalities were noted in a rat model of meningitis induced by infection with *T. brucei* (Quan et al., 1999). Also in case of induction of brain injury, CP response is similar, as observed in a mice model of traumatic brain injury (TBI). Using iodine tracer, an increase in BCSFB permeability was observed in injured animals, already 5 h after the stab injury. Moreover, when immunostainings of injured and control CP were compared, injured CP appeared shrunken (Johanson et al., 2011). In another model of brain injury, namely non-penetrative blast, ultrastructural changes of the CPE were observed 1 day after the injury, described

as intracellular spaces being greatly widened between shrunken CPE cells, accompanied with monocytes invasion in same time point (Kaur et al., 1996). In agreement with this, *in vitro* studies of the TBI effect on the BCSFB revealed widening of intercellular spaces between CPE cells shortly after the insult, and consequent invasion of monocytes (Szmydynger-Chodobska et al., 2012).

Interestingly, immune cells were localised in between CPE cells up to 7 days after the injury (Kaur et al., 1996). Nevertheless, at 21 and 28 days after the injury, morphology and function of BCSFB was comparable to control. These findings are in accordance to the results obtained in this study, indicating quick restoration of BCSFB integrity in days after the injection, and pointing out to the great importance of structural and metabolic stabilisation of BCSFB for normal brain functioning.

In the patients who suffered acute ischemic stroke, loss of BCSFB integrity was noted and severity of BCSFB disruption was linked to final infarct volume (Renu et al., 2017). Studies on animal models of experimental stroke confirm these findings, showing BCSFB breakdown already 1 h after reperfusion (Batra et al., 2010). In an animal model of hemorrhage, ultrastructural changes in CP, such as disintegration of normal epithelium and swollen mitochondria, were observed 24 h after the injury (Gram et al., 2014).

Interestingly, in a study performed on MS patients, no significant morphological changes were detected in CP, when compared to viral encephalitis or control brains, although on these very samples contribution of CP in neuroinflammatory processes in MS was shown (Vercellino et al., 2008). One potential explanation for this result could come from the fact that cells were only studied via hematoxylin-eosin staining, which was not precise enough to be able to perceive ultrastructural changes. However, in another study performed on postmortem CP tissue, downregulation of *Cldn-3* and *Cldn*-5, components of TJ, was noted in MS patients in comparison to healthy subjects (Kooij et al., 2014). Furthermore, in experimental autoimmune encephalomyelitis (EAE), an animal model of MS, morphological changes were visible in CP, with the tendency to

aggravate with disease progression. Authors stated that some CPE cells appeared shrunken, which coincided with the changes in distribution and expression of TJ components (Engelhardt et al., 2001). Moreover, migration of T cells through CP has been observed in the early stages of induction of EAE, as well as accumulation of granulocytes and myeloid cells in CP stroma prior to the onset of the disease (Engelhardt et al., 2001).

In the studies performed in Alzheimer's disease (AD) patients, as well as in patients with mild cognitive impairment (MCI), increase in BCSFB permeability is notable (Ott et al., 2018). Moreover, in a recent study performed on CP samples from AD patients, decrease in mRNA and protein expression of CLDN-5 was shown (Bergen et al., 2015). On the other hand, in transgenic mouse models of AD, increase in BCSFB permeability was not explicitly shown (Marques et al., 2013a). The possible explanation for this could be the fact that increase in BCSFB permeability is transient, and that identification of particular moment is challenging in chronic model of the disease. Moreover, in AD, the problem to identify events occurring early in the disease comes from the fact that AD is mostly not diagnosed before the clinical symptoms show, which presents a late time point. Since discussed changes in CP functionality occur before AD pathology becomes apparent, use of transgenic mouse models to study CP response to neuroinflammation is somewhat disadvantageous. Hence, an acute model, such as i.c.v. AβO injection gave us the opportunity to investigate events occuring in the initiation of neuroinflammation (Balusu et al., 2016a).

Regarding morphological changes of CPE cells, in both, AD patients and animal models, decrease in cell height, reduction in number of microvilli and mitochondrial density, and appearance of lipofuscin deposits and Biondi ring tangles were consistently noted (Marques et al., 2013a). In this study, lack of mitochondrial changes and absence of lipofuscin deposits and Biondi ring tangles, might be due to the short observation time or too low dose of AβO, however further studies might be needed in order to answer these questions.

The above findings support the hypothesis that in various brain diseases associated with neuroinflammation, including the model of i.c.v. AβO injection, an increase in BCSFB permeability is accompanied with loss of typical cuboidal shape of CP cells and decrease in expression of tight junction components. A similar response is observed, whether the stimulus is derived from the periphery or the CNS. Moreover, transient BCSFB opening, and the tendency to return to homeostasis, adds to the idea of the CP as important player in immune processes in the brain.

Besides the loss of BCSFB integrity, also **BBB disruption** has been found in many neurological diseases, and mostly accompanies neuroinflammation (Tietz and Engelhardt, 2015). In order to examine whether alterations at the BCSFB precede changes at the BBB itspermeability was also analyzed at the time point at which BCSFB integrity is significantly affected. This analysis revealed no significant change in BBB permeability 6 h after i.c.v injection of AβO (Brkic et al., 2015b). Similarly, Vandenbroucke *et al*., did not observe an increase in BBB permeability 8h after i.p. LPS injection (Vandenbroucke et al., 2012). However, in another study on LPS-injected mice, BBB disruption was noted only in the group administered with highest dose of LPS, 24 h after the injection. At the same time, sickness symptoms occurred at much lower doses, which revealed relative resistance to LPS-induced BBB breakdown. In the same study, BCSFB breakdown was found already at 4 h time point, preceding BBB breakdown (Banks et al., 2015). Nevertheless, it must be taken into account that LPS-induced BBB disruption is variable in relation to LPS dose and brain health, meaning that BBB integrity of an already diseased animal is more sensitive to systemic inflammation (Varatharaj and Galea, 2017). In the cortical contusion model of brain injury in rats, an increase in BBB permeability was noted 6 h after the injury (Shigemori et al., 2006). However, in the same model alterations in BCSFB permeability was not assessed. In stroke, BBB permeability alteration after focal ischemia is not immediately detectable in patients (Betz, 1996). Nevertheless, in MCAO, an animal model of stroke, permeability of BBB was observed to be increased 24 h after the insult. Moreover, in another model of experimental stroke it was shown that BBB

disruption occurs not before 2 days after reperfusion, which is long after increased BCSFB permeability was observed already 1 h after insult (Batra et al., 2010). In MS patients, it has been found that BBB pathology is present especially in the areas susceptible to development of new MS lesions (Cramer et al., 2013). Moreover, correlation between disease severity and BBB permeability has been observed in EAE (Fabis et al., 2007). However, no clear data point toward relationship between BCSFB and BBB opening in MS. Increase in BBB permeability is also found in aged individuals, occurring as an early event, especially in hippocampus, being one of the first areas of the brain to deteriorate in aging and AD (Montagne et al., 2015). Interestingly, a recent paper showed that an increase in BCSFB permeability in AD and MCI patients occurs prior to BBB disruption (Ott et al., 2018). Thus, in our model, one might suspect that BBB did not show increase in permeability due to the choice to test only early time points (6 h), and the time frame of the increase in BBB permeability might be interesting to address in further studies.

Since, it has been noted that BBB disruption results from the alteration in TJ components (Tietz and Engelhardt, 2015), we also studied TJs at the BBB in our AD model. The results show that *Ocln* and *Zo1* mRNA expression for remained unchanged 6 h after i.c.v injection of AβO, while *Cldn1* mRNA was slightly downregulated. Significant decrease in mRNA expression showed only *Cldn5* (Brkic et al., 2015b). Interestingly, in other AD studies, *Cldn-5* was also observed to be decreased on BBB (Bake et al., 2009; Lee et al., 2012). Studies performed in the TBI rat model revealed downregulation of TJs linked to BBB breakdown (Shlosberg et al., 2010). Similar results regarding downregulation of TJ components were found in stroke (Sandoval and Witt, 2008). Interestingly, in the model of focal ischemia, it has been observed that changes in BBB TJs are not immediate, but occur after hours of continuous decrease in blood-flow (Betz, 1996). Research done on postmortem brain samples from MS patients show abnormalities in TJ components distribution (Ocln, ZO-1), but no change was observed in AJ components (Kirk et al., 2003; Padden et al., 2007; Plumb et al., 2002). Moreover, in the EAE mouse model, ZO-1 and occludin, but not claudin-5 were found to be altered at the 24 h time point at the BBB (Banks et al., 2015). The above mentioned

results implicate that BCSFB increase in permeability is an early event in the initiation of neuroinflammation, occurring prior to BBB breakdown.

Since, morphological changes of CPE cells are accompanied, and even preceded by alternation of the functionality of the CP, in this study secretory activity of the CP was tested. One of the CPE-specific proteins is transthyretin (TTR), a molecule with a role implicated in the delivery of thyroid hormones to the brain (Richardson et al., 2015). No changes were found in *Ttr* mRNA expression in the CP 2 and 6 h after AβO i.c.v. injection, nor did TTR immunostaining of CP at the 6 h time point reveal any differences. In accordance to this, in a study performed on CP deriving from AD patients, in comparison to their controls, *Ttr* gene expression remained stable. However, reduced levels of TTR were found in CSF of patients with AD (Riisoen, 1988). The explanation to this phenomenon might come from the proposed protective role of TTR in clearance of amyloid beta from the brain (Alemi et al., 2016). A study performed on two different AD transgenic mouse models, also did not show any alteration in *Ttr* mRNA expression in CP in comparison to wt animals. Similarly, in a study done on mice administered with LPS, no differences in *Ttr* expression in CP were observed 72 h after the injection (Marques et al., 2007). Our results are in agreement with other papers that explore mRNA changes in CP after inflammatory stimulus, such as LPS, no changes in *Ttr* expression were observed (Marques and Sousa, 2015; Marques et al., 2009b). Altogether, one might speculate that, although mRNA for *Ttr* constitutes 50% of all mRNAs synthesized in CP, this protein does not play an important role in the inflammatory processes (Zheng and Chodobski, 2005). However, prospective studies in this field should be additionally performed.

Through adaptation of its secretory activity, the CP responds to different pathological conditions, from injury to diseases. For these purposes, CP secretes in the CSF numerous growth and transcription factors, including BDNF, IGF, and NT-3 (Zappaterra and Lehtinen, 2012). Although the role of neurotrophic factors, such as BDNF, in the developing brain is well known, also their role in the adult brain has been implicated in proliferation, cell survival and synaptic plasticity (Binder and Scharfman, 2004). Moreover, therapeutic potential of CP derived BDNF was

proposed (Deng et al., 2016; Huang et al., 2014). In this study, significant downregulation of *Bdnf* gene expression in CP, was observed as well as a drop in concentration of BDNF in CSF 6 h after i.c.v. injection of AβO. The question remaining open for further studies is whether this decrease in BDNF levels solely derives from diminished secretory activity of CP. Interestingly, it was already shown that i.c.v. injection of AβO was able to induce a decrease in BDNF concentration in the cortex and hippocampus, and in the same study authors have found that in APP/PS1, transgenic mouse model of AD, BDNF gene and protein expression in the cortex and hippocampus was decreased in comparison to control in both, 3 and 9 months old animals (Xia et al., 2017). Moreover, decrease in BDNF concentration was also linked to cognitive deficits in AβO i.c.v. injected rats (Zhang et al., 2015). In the study performed for this doctoral thesis, cognitive impairment was observed as decline in STM and LTM in mice after AβO i.c.v. injection (Steeland et al., 2018). In a study performed by another group the same effect on long term memory impairment after i.c.v. injection of AβO was noted (Balducci et al., 2010). Another possible mechanism through which cognitive impairment might occur is through direct effect of AβO on inhibition of long term potentiation in the hippocampus of mice (Townsend et al., 2006), since it has been showed that AβO is potent enough to initiate the pathological processes that finally give the characteristic clinical picture of AD, with one of the hallmarks being the memory impairment.

Similar results regarding decrease in BDNF levels are found in different brain diseases associated with neuroinflammation. In an animal model of stroke, CP-derived BDNF was found to be able to reduce infarction size and ameliorate behavioral impairment on 2nd and 3rd day after a transplantation of a healthy porcine CP in the rat that suffered from middle cerebral artery occlusion (MCAO) (Borlongan et al., 2004). Moreover, in TBI, neurotrophins derived from CP were also implicated in the recovery processes after the injury (Johanson et al., 2011). In AD patients, in comparison to MCI patients and healthy controls, reduction of BDNF levels in CSF was also observed (Forlenza et al., 2015).

Besides increased permeability and underlying morphological alterations, it has been shown that the CP responds on inflammatory signals from both the CNS (Batra et al., 2010; Sharma et al., 2010; Simard et al., 2011) and periphery (Marques et al., 2009a; Marques et al., 2009b; Marques et al., 2007) through production and secretion of different cytokines and that this response has been observed after introduction of various toxic molecules, infection with pathogens, as well as in different diseases of the brain. In general, as well as in CP, in order to amplify the response to the detected inflammatory stimulus, various cytokines are secreted, causing attraction of additional immune cells to the site of inflammation (Balusu et al., 2016a).

In this study, significant elevation of the gene expression for proinflammatory cytokines, *Il1β*, *Il6* and *Tnf,* was observed in the CP already 2 h after AβO i.c.v injection, as well as in hippocampus (Steeland et al., 2018). Also CSF protein levels of IL-1β, IL-6 and TNF, as well as of several other cytokines, IL-1α, IL-3, IL-10, IL12-p40, IL-13, IL-17, Rantes, MIP-1α, MIP-1β, MCP-1, IFN-γ and GM-CSF were significantly elevated 2 h after the injection. These changes were even more prominent 6 h after AβO i.c.v injection, illustrating an increase in inflammatory signaling (Brkic et al., 2015b). Previously, it was published that in LPS stimulated mice, mRNA levels of *Il6* are significantly upregulated in the CP already 4 h after the injection, while in CSF, significant increase in protein levels of IL-6, MIP-1α, MIP-1β, MIP-2 and MCP-1 was measured 8 h post injection (Vandenbroucke et al., 2012). Similar results were observed in another study with goats injected with LPS, where an increase in cytokine and chemokine levels (MCP-1, MIP-2, and IL-6) in CP was observed 4 h after injection (Salvesen et al., 2017). Furthermore, changes in the whole CP transcriptome was observed upon repeated peripheral stimulation with LPS, especially regarding the cluster of immunoregulatory and extracellular matrix remodeling genes (Marques et al., 2007). In the same study, authors noted that changes in CP mRNA levels of *Il1β* and *Tnf* peak already 1 hour after LPS induced systemic inflammation, staying still significantly elevated 3 days after the injection. In addition, at 1h time point they observed an increase in TNF in CSF. Moreover, in patients suffering from sepsis,

significantly increased levels of proinflammatory cytokines in CSF (IL-6, IL-8, IL-18 and TNF) were associated with disease severity and mortality (Chaudhry et al., 2013). Earlier, it was believed that in sepsis, cytokines from blood transmit the inflammatory message to CNS by two distinct ways: through the vagus nerve and through the circumventricular organs. Latest research showed that primal responders in the neuroimmune response to LPS i.p. injected in mice are barrier cells that secrete cytokines in CSF, leading to activation of hippocampal astrocytes that further produce cytokines, resulting in the amplified neuroimmune reaction (Danielski et al., 2018; Hasegawa-Ishii et al., 2016). In patients suffering from TBI, an increase in various proinflammatory cytokines, especially TNF, IL-1β and IL-6 has been observed in CSF (Buttram et al., 2007). It has been indicated that the secretion of these proinflammatory cytokines by the CP, aids in trafficking of leukocytes through CP to the brain. Moreover, in a study using the rat model of TBI, induction of cytokines and chemokines production was observed in CP. *Tnf* and *Il1β* gene expression was observed to peak 6 h after the impact on the brain, while gene expression levels of *Mip-2* and few other chemokines were also significantly upregulated in the same time point. Additionally, these chemokines were implicated in the neutrophil invasion associated with TBI (Szmydynger-Chodobska et al., 2009). Similarly, in another study, the role of MCP-1 in TBI has been established, a prompt elevation in CPE after the insult which induced chemotaxis of leukocytes (Szmydynger-Chodobska et al., 2010). After ischemia, initiation of inflammation is an important step in the progression of ischemia-reperfusion injury. It has been shown that in CSF protein concentration of several cytokines such as TNF, IL-1β and IL-6 increase in early hours after stroke in both, humans and animal models (Lambertsen et al., 2012; Whiteley et al., 2009). Also, in a rabbit model of intraventricular hemorrhage, increase in gene expression for *Il-1β*, *Tnf*, *Mcp-1*, *Il-8* and *Il-6* was observed in the CP at 24 and 72 h timepoints after the insult (Gram et al., 2014). In MS, inflammation in the CP was observed persistently in diseased patients, in comparison to their healthy controls (Vercellino et al., 2008). Similarly, transcriptome changes and increase in gene expression for cytokine and chemokines pathways were observed in CP in all phases of the EAE (Marques et al., 2012). Interestingly, in the same animal model of the disease,

inflammation in the CP was observed to precede formation of demyelinating white matter lesions, which presents the hallmark of the disease (Engelhardt and Sorokin, 2009). In AD patients, an increase in protein expression of several proinflammatory cytokines, such as TNF and IL-1β was found in CSF (Llano et al., 2012; Tarkowski et al., 2003). Moreover, the results of the research that began for this thesis further stressed the importance of TNF, as one of the key inflammatory mediators in the CP in AD (Steeland et al., 2018). In a recent study, authors compared gene expression in CP of advanced AD and control patients, and noted upregulation of genes for various cytokines, predominantly interleukines (Stopa et al., 2018). Similarly, increase in expression of proinflammatory cytokines was noted in CP transcripts of AD patients examined from the Brown-Merck Gene Expression Omnibus database (Kant et al., 2018). Moreover, studies performed *in vitro* and *in vivo* in animal models, implicate that inflammation appears before amyloid plaques and neurofibrillary tangles. These findings are in accordance to the Rotterdam study, where increased levels of proinflammatory cytokines were detected in plasma of diseased patients before clinical signs of the disease (Engelhart et al., 2004; Floden et al., 2005).

Cytokines exert a plethora of events that can further aggravate inflammatory response. They can induce expression of leukocyte adhesion molecules that trigger infiltration of immune cells into CNS, and they can active production of other cytokines that can further spread the inflammatory signal, ultimately activating other immune cells, such as microglia (Turner et al., 2014). Moreover, proinflammatory cytokines, such as TNF and IL1β, are showed to be able to activate signaling pathways that, through transcription factors, such as AP-1 or NFkB, can initiate transcription of *e.g.* matrix metalloproteinases (MMPs). When active, MMPs can proteolytically cleave other MMPs, therefore activating them (Serrano-Pozo et al., 2011). As MMPs can also cleave various other molecules, including cytokines, chemokines, death receptors and growth factors, they play an active role in inflammatory processes and contribute to the perpetuation of inflammation (Berg et al., 2018). MMPs can also cleave ECM and TJ components, leading to the increase in permeability of the barriers (Schubert-Unkmeir et al.,

2010). In the literature, scarce data exist about MMP effect on BCSFB in the initiation of neuroinflammation (Brkic et al., 2015a). Previously, our lab showed that MMP-8 is able to contribute to an increased BCSFB permeability in LPS-induced systemic inflammation (Vandenbroucke et al., 2012). Thus, in the study on i.c.v. injection of AβO in mice, contribution of MMPs to the initiation of neuroinflammation by increasing BCSFB permeability was examined. This study revealed that i.c.v. injection of AβO induces an increase in *Mmp3*, *Mmp8* and *Mmp9* gene expression in the CP already 6 h after disease induction. Moreover, MMP activity in CSF was found to be significantly enhanced after AβO injection (Brkic et al., 2015b). Contributing further to the hypothesis of the pivot role of MMPs in inflammatory processes, previously the lab revealed that the application of broad spectrum MMP inhibitor brought full protection against LPS-induced death (Vandenbroucke et al., 2012). Next, it was hypothesized that injection of broad spectrum inhibitor might also block MMP activity upon i.c.v. injection of AβO, and rescue the AβO-induced loss of BCSFB integrity. Our results indeed showed that injection of GM6001, a broad spectrum MMP inhibitor, together with AβO, was able to reverse MMP activity to control levels. Moreover, this was also associated with a reduction in the AβO-induced BCSFB leakage. Similar effects of AβO through MMP activity was earlier shown to be associated with BBB breakdown. In endothelial cell culture, increase in BBB permeability was shown to be due to downregulation of ZO-1 levels, accompanied with increase in MMP-9 activity. In 8-month- old 5xFAD transgenic mouse model of AD, increased immunoreactivity of MMP-9 was shown near cerebral capillaries. In addition, broad-spectrum MMP inhibitor was shown to be able to alleviate AβO-induced BBB disruption (Kook et al., 2012). Although several MMPs have been implicated in proteolytic cleavage leading to barrier openings, the role of MMP-3 in this AβO-induced BCSFB permeability was further studied. While MMP-3 deficient mice showed a reduced AβO-induced BCSFB permeability, MMP-3 deficient mice still showed some AβO-induced loss of BCSFB integrity, pointing towards contribution of additional MMPs to the increase in BCSFB permeability. Similarly, in one of the studied in the host lab, injection of LPS in MMP-8 deficient mice caused significantly less mortality

compared to wild type mice, pointing towards the importance of other MMPs in this process (Vandenbroucke et al., 2012).

In other studies of MMPs in sepsis, it has been found that in patients with septic shock mRNA expression of *Mmp8* is increased especially in non-surviving patients (Nakamura et al., 1998) and plasma samples collected from sepsis patients revealed that levels of MMP-9 were significantly elevated in diseased patients in comparison to control (Muhl et al., 2011). Moreover, MMP-9 deficient mice were resistant to endotoxin induced shock (Dubois et al., 2002). In accordance to above findings, MMP-9 and MMP-3 were implicated in BBB disruption in LPS injected mice (Gurney et al., 2006; Mun-Bryce et al., 2002). Also in many other diseases associated with neuroinflammation, MMPs were shown to play important roles in the opening of CNS barriers and in the initiation of neuroinflammation. In TBI, increased MMP-9 and -3 levels were found in human CSF 24 h after arrival to the hospital (Grossetete et al., 2009). Using the cortical contusion model of brain injury in rats, MMP-9 has been implicated in BBB disruption which occurred 6 h after injury, while MMP-9 was shown to be upregulated as early as 3 h post injury with a peak at 18 h. Others showed that the broad-spectrum MMP inhibitor, GM6001, was effective in mitigating the consequences of the injury, such as brain edema and BBB disruption (Shigemori et al., 2006). So far, no data were published on the role of MMPs in BCSFB breakdown in TBI, but preliminary data from our laboratory show a major contribution of MMPs in the increased BCSFB permeability in the stab wound injury mouse model (unpublished results). In studies done on patients with acute ischemic stroke, MMP-9 was even proposed as biomarker of the disease, since it is found to be significantly upregulated in plasma of the patients. Moreover, in the same study it was observed that MMP-9 levels correlated with infarct volume, as well as with final outcome (Ramos-Fernandez et al., 2011). Moreover, MMP-9 was observed to be upregulated in CSF in humans 24 h, and in animals already 3 h after stroke (Heo et al., 1999; Martin et al., 2012) and has been implicated in BCSFB breakdown using an animal model of experimental stroke (Batra et al., 2010). Additionally, MMP-9 deficient mice subjected to transient focal ischemia showed less BBB

leakage and in infarct volume compared to wild type mice (Asahi et al., 2001). In MS, upregulated levels of several MMPs, especially MMP-9, were found in serum, brain and CSF samples from diseased patients (Yong et al., 2007). Interestingly, acute attacks of MS were observed to be accompanied with loss of BBB integrity and increased MMP-9 CSF levels (Rosenberg et al., 1996). In a rat model of EAE, migration of leukocytes, a characteristic of an acute attack in MS and a consequence of opening of the CNS barriers, is shown to be MMP dependent (Agrawal et al., 2006). Use of broad spectrum MMP inhibitors and genetically engineered mice deficient for MMPs showed reduction in permeability of BBB and fewer clinical symptoms (Yong et al., 2007). As far as the link between MMPs and AD is concerned, in order to be able to separate AD from vascular dementia, Bjerke *et al*. suggested that levels of MMP-9 and tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) in CSF can be used as biomarkers of AD (Bjerke et al., 2011). Moreover, other authors observed increased plasma and CSF levels of MMP-3 in AD patients (Horstmann et al., 2010). Altogether, these data point to a major role of MMPs in the inflammatory processes, as well as in the increase of BBB and BCSFB permeability through cleavage of TJ components, hence greatly contributing to initiation and in some diseases perpetuation of neuroinflammation.

Another important type of cell-to-cell communication in physiological and pathological conditions is conveyed through **EVs** (Paolicelli et al., 2018). Due to its position in the brain and the role in CSF production, the CP is perceived as an important location of EVs production due to its ability to secrete EVs into the CSF (Holm et al., 2018). This enables EVs to be spread throughout the brain, target different cells such as microglia and astrocytes, deliver their cargo, induce an inflammatory response, and hence contribute to neuroinflammatory processes. When the work described in this thesis was performed, no data were available on the production of EVs in the CP upon inflammatory triggers such as i.p. injection of LPS or i.c.v. AβO injection. Herewith, it was shown that upon LPS administration the number of particles increased in the CSF as soon as 2 h after i.p. LPS injection. It was noted that this is associated with upregulation of *Cd63*, a marker of EVs (Balusu et al., 2016b). This led us to speculate that the increased number of

particles in the CSF, including EVs, is at least partially derived from an increased secretion of EVs by the CPE. Similar results were obtained upon i.c.v. injection of AβO. As soon as 2 h after i.c.v. injection, increased amount of particles could be detected in the CSF. Moreover, it was again observed upregulation of *Cd63* gene expression in the CP. The amount of EVs peaked 24 h after i.c.v injection which is in correlation with the observed BCSFB permeability. One week after AβO injection, both the amount of EVs and the BCSFB permeability were back at basal levels. In agreement with these results, the role of EVs has been implicated in various brain diseases associated with neuroinflammation. In sepsis, an increase in number of EVs and their detrimental effects have been described (Essandoh et al., 2015). In a study performed for this thesis, on two animal models of sepsis, LPS injection and cecal ligation puncture, pretreatment with GW4869, an exosome inhibitor, resulted in less EVs in the CSF. Interestingly, this was accompanied with a reduction in the amount of proinflammatory cytokines, TNF, IL-1β and IL-6 in serum of treated animals in comparison to control mice, already 12 h after the initiation of the disease (Balusu et al., 2016b). In TBI, increased number of EVs was seen in CSF, and their potential as biomarkers of injury was proposed (Manek et al., 2018). In MS patients, especially during relapse, an increase in EVs was noted compared to patients with chronic disease or healthy individuals (Alexander et al., 2015a). Interestingly, orally-administered drug for this disease was shown to induce a decrease in number of EVs in the CSF (Selmaj et al., 2017), and the mice in which EV production was inhibited were protected from EAE (Verderio et al., 2012). The same study explained how EVs transmit an inflammatory message *in vitro* and *in vivo*, inducing a dose dependent increase in proinflammatory cytokines produced by the recipient cells. Also, the authors showed that the origin of those EVs is most probably CP, and not parenchymal microglia. In particular, EVs are shown to be able to stimulate endothelial cells, leukocytes, monocytes and glial cells, and to induce secretion of cytokines (Selmaj et al., 2017).

The effect of EVs on their target cells depends on their cargo. MiRNAs are of specific interest as cargo, since they are able to modulate gene expression in the recipient cells. Since miR-155 was able to stimulate LPS-induced inflammation in

mice (Alexander et al., 2015b) and activate innate immune response (O'Connell et al., 2007), the presence of miR-155 in our two models was analyzed, taking into account the fact that it was also found as cargo in EVs (Bala et al., 2015). Upon i.p. LPS injection, a significant increase in miR-155 expression in the CP 6 h after disease induction was observed (Balusu et al., 2016b). Similarly, miR-155 expression was also upregulated in the CP 6 h after i.c.v. AβO injection. In a study using an animal model of TBI, EVs from injured animals were able to activate microglia when administered *in vitro,* and to induce secretion of IL-1β and miR-155, which contributes to the induction of neuroinflammation after the injury (Kumar et al., 2017). Also another study found elevated miR-155 levels in the brain in an animal model of TBI, pointing to the importance of this proinflammatory miRNA in the aggravation of the outcome after the injury (Harrison et al., 2017). Also in MS and EAE miR-155 has been shown to amplify expression of proinflammatory genes, contributing to activation of immune cells and microglia and increasing BBB permeability (McCoy, 2017). In AD patients, increased levels of miR-155 were noted in CSF (Olivieri et al., 2013). Besides the role of EVs as transporters of Aβ in the brain of AD patients (Gupta and Pulliam, 2014), miR-155 was also shown to play an important role in activation of innate immune response and overall contribution to inflammation in the brain in AD (Guedes et al., 2014; Song and Lee, 2015).

Since, one of the targets and sources of EVs regarding complex communication in inflammatory processes are microglial cells, the research performed for this thesis aimed at determining whether activation of microglia precedes or follows secretion of proinflammatory cytokines from the CP. Microglia mostly reside in the brain in so called 'resting' state, although it is constantly employed in brain surveillance. Upon activation, microglia changes morphologically and functionally, and starts producing either pro- or anti-inflammatory cytokines (Kraft and Harry, 2011). In this study the number of active microglial cells around cerebral ventricles remained the same 6 h after Aβ 1-42 i.c.v. injection (Brkic et al., 2015b), whereas increase in proinflammatory cytokines in CSF was notable at the same time point, one might speculate that observed

increase in proinflammatory cytokines in CSF is of a CP origin, rather than from microglia. Further studies in later time points need to be performed in order to examine timeframe in which microglial cells become activated, since in the literature microglial activation by AβO has been reported in AD (Maezawa et al., 2011). Interestingly, an *in vitro* study on microglial cells prepared from embryonic human telencephalon tissue, showed a similar response after stimulation with LPS or Aβ, manifested as increased gene expression of proinflammatory cytokines (TNF, MIP1α, MIP1β, and MCP-1) (Lee et al., 2002). So, besides proinflammatory cytokines deriving from CP, AβO can also activate microglial cells. Although there is a great abundance of work on the role of microglia in diseases associated with neuroinflammation, which are not the subject of this thesis, and thus will not be mentioned here, there is scarce data in the literature on relation between CP and microglia in the initiation of neuroinflammation. Thus, further studies are needed in order to address this issue, and reveal the communication of these two important players in the initiation of neuroinflammatory processes.

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To be able to harness all the possibilities of human organism to fight against the diseases, one must first understand the events occurring in the development of the disease and the mechanism of reaction of human organisms. Until recently, the CP tissue was largely neglected in research, and only lately its important role in the initiation of neuroinflammation is starting to be revealed. Determining the way that the CP responds to various peripheral or CNS inflammatory stimuli and discovering similarities, helps to understand the thin balance between beneficial and harmful contribution to resolution of neuroinflammation. Moreover, it helps us to identify how to mitigate spreading of the inflammatory message throughout the brain. Findings from our laboratory analyzing the CP response to i.p. LPS injection, proved as crucial and very insightful and made a great starting point to examine events occuring on BCSFB in the initiation of neuroinflammation.

Moreover, using novel model of i.c.v. AβO injection enabled us to examine early events occurring in cascade of inflammatory processes in AD. Comparison of CP response experimentally in two different animal models of disease associated with neuroinflammation enabled us to observe uniformity of inflammatory response of BCSFB, with stimuli either deriving from periphery or from central nervous system. This uniformity reflects on increase in secretion of various proinflammatory cytokines, which among other effects initiate MMPs production, secretion and activation, leading to an increase in BCSFB permeability and alteration in morphology of CPE cells. Moreover, this response initiates secretion of EVs which further spread inflammatory signals throughout the brain.

Finally, after closer understanding of the mechanisms involved in BCSFB opening and initiation of neuroinflammatory processes, further studies are needed to reveal whether the BCSFB has a role in perpetuation of inflammation, and how the biological role of the CP can be harnessed to contribute to the resolution of inflammation.

6. Conclusions

The results of the study on structural and functional changes of the CP in response to neuroinflammation in mice revealed a similar response to i.c.v. AβO injections compared to i.p. LPS injection or other inflammatory stimuli. Taking into account all data collected and analyzed in this study, the following conclusions can be made:

(1) BCSFB permeability is increased upon i.c.v. AβO injection, resulting from the loss of typical cuboidal morphology of CPE cells and decrease in expression of tight junctions components. This effect was very quick, but transient.

(2) BBB permeability remained unaffected 6 h after i.c.v. AβO injection.

(3) Mice showed short and long term memory impairment after i.c.v. AβO injection.

(4) I.c.v. AβO injection is associated with:

unaltered *Ttr* gene expression and TTR protein production in the CP;

downregulation of *Bdnf* gene expression in CP and BDNF levels in CSF;

upregulation of gene expression for various cytokines, and increased secretion in the CSF;

increased *Mmp* gene expression in the CP, and increased MMP activity in the CSF.

(5) Contribution of MMPs to increased BCSFB permeability was shown through i.c.v. injection of broad spectrum inhibitor, GM6001, together with AβO, which prevented AβO-induced BCSFB permeability. Moreover, also MMP3 deficient mice showed a reduced increase in BCSFB permeability upon i.c.v. AβO injection, which confirms the role of MMPs in these processes.

(6) Both i.p. LPS and i.c.v. AβO injection induce an increase in number of particles in the CSF and an increase in gene expression of EV markers in the CP.

(7) The number of particles in the CSF correlates with BCSFB permeability, which returns to control levels one week after i.c.v. AβO injection.

(8) Both i.p. LPS and i.c.v. AβO injection induce an increase in miR-155 levels in the CP.

(9) I.c.v. AβO injection doesn't significantly activate microglia.

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Marjana Brkić was born on 23.12.1984. in Belgrade, where she went to elementary school and high school. She enrolled Faculty of Biology in 2003, where she graduated in 2009. From the Molecular biology and physiology group, with 9,52 average grade mark. She received highest mark (10) on graduation exam. Afterwards, in 2009. she enrolled to Master studies at the Faculty of Organisational Sciences, group Management and organisation - Technical and Technological Sciences, where she graduated in 2012. Doctoral studies at the Faculty of Biology, she enrolled in 2012. Also, she was employed at Department of Neurobiology, Institute for Biological Research „Siniša Stanković" (IBISS), University of Belgrade from 2012. to 2017. Marjana's research at IBISS, was supported by project financed by Ministry of Science, Education and Technological Development of Republic of Serbia, named "Brain plasticity in aging: effect of dietary restriction and anesthesia" (project number ON173056, Ministry of Science, Education and Technological Development, PI Selma Kanazir). In 2013, Marjana spent six-months long research stay through Basileus IV project, Erasmus Mundus Action 2 grant at VIB - UGent Center for Inflammation Research, University of Ghent, as a PhD exchange student. In 2017. Marjana enrolled to Doctoral studies at the University of Ghent.

Her thesis is done under joint supervision of Prof. Dr. Roosmarijn Vandenbroucke and Dr. Selma Kanazir.

Marjana is an author of 2 and a coauthor of 7 publication in leading international journals, as well as numerous congress publications.

Прилог 1.

8. Изјава о ауторству

Потписани-а **Марјана Бркић**

број индекса **Б3043/2012**

Изјављујем

да је докторска дисертација под насловом

Значај структурних и функционалних промена хороидног плексуса миша у иницијацији неуроинфламације

- резултат сопственог истраживачког рада,
- да предложена дисертација у целини ни у деловима није била предложена за добијање било које дипломе према студијским програмима других високошколских установа,
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У Београду, 15.06.2019.

Прилог 2.

9. Изјава о истоветности штампане и електронске верзије докторског рада

Име и презиме аутора **Марјана Бркић**

Број индекса **Б3043/2012**

Студијски програм **Биологија**

Наслов рада **Значај структурних и функционалних промена хороидног плексуса миша у иницијацији неуроинфламације**

Ментори **др Селма Каназир и др Roosmarijn Vandenbroucke**

Потписани/а **Марјана Бркић**

Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла за објављивање на порталу **Дигиталног репозиторијума Универзитета у Београду.**

Дозвољавам да се објаве моји лични подаци везани за добијање академског звања доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

Потпис докторанда

У Београду, 15.06.2019.

Прилог 3.

10. Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић“ да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

Значај структурних и функционалних промена хороидног плексуса миша у иницијацији неуроинфламације

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигитални репозиторијум Универзитета у Београду могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

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